

Confused comment on Warnock

The British House of Commons has had a muddled debate about the regulation of in vitro fertilization. The British government should devise more liberal legislation than was urged last week.

DAME Mary Warnock's report on *in vitro* fertilization and related matters seems to have had a better reception from the general press than from the British House of Commons, which spent last Friday (22 November) debating the issues that had been raised. By legend, this would have been one of those occasions when the British parliament should have been at its best. With nothing to be decided, and with no compulsion on party managers to dragoon their members through some predetermined voting booth (called a "division lobby" at Westminster), Members of Parliament with a philosophical turn of mind should have been free to take a broad view of an important set of issues, giving the government some durable help with the design of the legislation that it is committed to produce. In the event, the discussion was down-at-heel, dominated by complaints that the Warnock Committee failed to define the point in the development of an embryo at which life may be said to begin, by expressions of horror at what people in laboratories were about and by some simple misunderstandings that would be laughable if they were not held by those who will be responsible for whatever legislation eventually emerges.

It is inevitable that liberal opinions should be drowned in such a clamour. Even so, the view that the practice of *in vitro* fertilization is an important part of the treatment of infertility was strongly put. The case for research with living early embryos was put, but unavoidably made to seem simplistic by the apparent need to make its importance tangible by guessing at what the outcome might be. Surrogate motherhood won only the most tepid advocacy — most people are against it. The House of Commons seemed not to be especially downcast at the reminder (which Warnock vividly provided) that it has done nothing, these past twenty years, to deal with the legal problems occasioned by the now widespread practice of artificial insemination (where, under British law as it stands, such a child is illegitimate and should formally be adopted by its mother's husband, and where Warnock recommended automatic legitimacy but also disclosure of the method of conception at the child's majority). In the circumstances, the most memorable feature of the debate was that those in favour of the proposition that embryos are living persons from the instant of conception included both Mr Ian Paisley, the Ulster Unionist, and Mr John Hume, the leader of the Social Democratic Labour Party in Ulster — inveterate opponents on almost every other issue. The danger only barely concealed by the debate, in which the government promised that it may be possible to find administrative devices for regulating Warnock matters in the spell before legislation can be devised, is that some MP will jump in with a private member's bill drawn so emotively that nobody will dare oppose it.

What the House of Commons should have understood last week, and what the British government must now appreciate, is that there is a world of difference between the novel techniques in human embryology that are already parts of medical practice and the opportunities that have arisen in research. That the use of novel medical techniques should be regulated is not, of course, unusual and is not, in principle, objectionable. In Britain as almost everywhere, both abortion and (further from the clinic) adoption are regulated by law. Warnock asked that those offering *in vitro* fertilization should be registered, which is fair; there is also a case for asking that data should be securely logged, on which the committee was ambiguous. The committee also

agonized about surrogacy, concluding that the practice is always undesirable but that surrogacy with a commercial element is frankly unacceptable, and must be made illegal. Mr Norman Fowler, the Secretary of State for Social Services, warned those within earshot that surrogacy with payment may already be illegal under the British adoption laws as they stand. But even that argument is not as simple as it sounds, for there is an important difference between what may seem the sale of intact people and the prearrangement of a genetically known birth. Even on this contentious point, the British government should shy away from outright bans, as one of the minority reports of the Warnock Committee asked.

Research is quite a different kettle of fish. The Warnock Committee recommended licensed research with embryos no older than 14 days. The weakness of that position is that the time-limit is arbitrary. Moreover, the case for research on early embryos is necessarily sketchy — it is the old difficulty that people cannot tell what they will find until they look. The sensible course of action for the government to follow is to accept the Warnock proposal for a committee to oversee the conduct of all research projects proposed, much along the lines of the committees that have regulated recombinant DNA research in different places, but with the added proviso that a project should be shown not merely to be harmless but potentially valuable. The time-limit should be dropped. And the proceedings of such a committee should not merely be public but publicized. Then even the House of Commons might understand why the opportunities in this field are exciting and important. □

Bettering farm support

The Reagan administration should give agricultural research its due.

AS the Reagan administration began its curious effort last week to find budget cuts that will eliminate a \$200,000 million a year deficit without raising taxes, one of its first discoveries was the \$10,000 million or so spent each year to keep farmers in business. According to the "supply-side" dogma that still seems to grip everyone in the administration (except perhaps David Stockman, director of the Office of Management and Budget), supply creates its own demand; remove restraints on production (that is, government intervention), and everything will work just fine. Hence the not-so-surprising talk of doing away with the 50-year-old strategy of farm price supports in favour of what is being called a "market-oriented" approach.

The Reagan administration is not renowned for its grasp of history, so a quick refresher course on what happened the last time the United States tried *laissez faire* in farming is perhaps in order. Above all, it is worth remembering that throughout the 1920s, it was not just US farmers but farmers everywhere who struggled with chronic overproduction. During that decade of booming prosperity for business and industry, the farm economy was deathly ill. The prices of commodities, always subject to cycles of boom and bust, swung dramatically; the price of wheat, for example, fell from \$3 a bushel to \$0.30. By 1932, when the depression had finally struck the rest of the economy, the prices farmers received for their crops compared with the prices they had



to pay for the goods they consumed had fallen to half that of 1914. In 1929, more than a quarter of US farm income came from exports, again now talked of as the salvation of the US farmer. But then, far from being a salvation, export dependence precipitated further disaster when the export market collapsed.

Overproduction is still a chronic problem. And if, now, imbalance of general trade has at least provided Europe and Japan with the US currency with which to purchase US goods (not the case in the 1920s), the potential customers' restraints on trade to protect their own farmers and the overpriced US dollar are at least equal barriers. It is difficult to see how overproduction of US farm commodities can create its own demand in these artificial circumstances.

There is nevertheless much that could be done to reform the US price support system. The administration is looking in the right place, if in the wrong spirit. Whatever its ambitions now, the administration will probably not be able to eliminate the system of price support, which has at least served well to smooth out the natural cycles of boom and bust. The chief mechanism is that of loans granted to farmers at harvest time against crops as collateral; if the farmer is unable to sell his crop at a price equal to or better than the loan price, he has the option of defaulting, in effect selling his crop to the government. This prevents a price-depressing glut at harvest time and removes a minimal amount of overproduction from the market. But there is little justification for maintaining the expensive programmes that amount to all-out production incentives. Any rational farm policy must put a premium on greater efficiency rather than greater production. That is simply not the case now, when farmers find the best and, indeed, often the only avenue to increased income is expansion.

The present approach to agricultural research can only exacerbate these structural failings of the farm programmes. The emphasis on applied, production-oriented, short-term results brought about by the anachronistic formula-fund system of agricultural research can only encourage the overproduction mentality. By every estimate, US agricultural research is woefully undersupported, yielding a return on investment many times greater than the norm for other businesses. The absence of peer review in the award of most research grants ensures that special interests have a disproportionate say in the choice of research priorities, exacerbating the short-term view of many researchers in the land-grant college system.

Yet a greater investment in fundamental research, particularly plant molecular biology, could lead to substantial reductions of production costs, for example by reducing or eliminating the need for energy-intensive inputs such as pesticides and fertilizers. The \$46 million that Congress managed to appropriate this year for research is a sound first step, but far less than private corporations are spending in this field. Now, within the overall context of US farm policy, the time has come to acknowledge that agricultural research is too important to be left to the vagaries of share-the-wealth formula funding, and more relevant to the long-term stability of the farm economy than the narrow pursuit of increased production that now passes for a research strategy. □

European initiatives

The European Science Foundation has had ten modest years. Now it needs to be more daring.

THAT it is high time that something more was done about the organization of science in Europe is beyond dispute. What perplexes people is to know with some clarity what should be done. Lord Flowers, the first president of the European Science Foundation, canvassed one modest set of proposals at the tenth anniversary meeting of the foundation in Strasbourg last week. In a public lecture on "the first twenty years" (a joke intended to encompass both the formal lifespan of the foundation and its gestation period), Flowers rightly claimed on behalf of the foundation a string of notable achievements in the past few decades, among which (just now) the most conspicuous is the plan for building a powerful source of synchrotron radiation in

Western Europe (see *Nature* 1 November, p.3 and 15 November, p.189). Properly, however, Flowers boasted of the way in which the foundation has been asked by the Council of Europe to carry out a study of the ways in which the mobility of scientists in Europe might be increased and he urged that the British government's last-minute proposal at last month's European meeting in Paris, that there should be a European academy of some kind, should be taken more seriously than its half-baked character would otherwise ensure. Persuasively, he argued that an institution such as the European Science Foundation, which is essentially a forum in which European grant-making agencies can put their heads together over common problems, and think of solving some of them, must run the danger of losing touch with the individual researchers on whom its success must depend.

Nobody in Europe will quarrel with a word of this, at least so far as it goes. Lord Flowers, the chief architect of the foundation, is understandably wedded to the notion of organic growth in European collaboration. He and others were also at the outset convinced that the creation of a body such as the European Science Foundation beneath the umbrella of the European Communities would be disastrous, however large the annual budget. That decision was without question correct. Freedom from the Commission's tutelage has allowed the foundation to draw its membership from all over Europe, not simply from that part in which the European Commission's legal writ runs. More important, the foundation has not found itself in the invidious position of having to look for solutions to the short-term and often artificial problems that the Commission repeatedly singles out as urgent. If it had accepted the Commission's offer of financial support, the foundation would long since have found itself tackling the impossible task of hammering out, or even carrying out, a research programme to make European computers better than other people's, or to resolve the endless (and intrinsically political) conflict about the basis on which environmental regulations should be set. Instead, the foundation has been able to tackle the tasks at which its chance of success was reasonably good. The long-term goal is the encouragement of a sense of community within European science. With the passage of time, the argument seems to run, the scientific community will itself determine how a European scientific community should be organized.

If there were the whole of time to play with, such a policy would make good sense. But the successful pursuit of the ideal is possible only within an ideal environment. In Europe as it really is, the problems afflicting the scientific communities of at least half a dozen states are too urgent to be allowed to wait. When European research institutes such as the European Organization for Nuclear Research (CERN), rightly held out as successes for European collaboration, are themselves threatened by the possible defection of member states such as Britain, when plans for collaborating in other international research programmes (such as deep-sea drilling) are frustrated by the general shortage of funds and when it seems likely that important pieces of nationally-built scientific equipment will be wasted because their builders (the British research councils in particular) can no longer afford to use them properly, there is a strong European case for some more robust mechanism for mutual support in times of trouble. For its own sake, the European Science Foundation does not want to be hurried. But that may be too great a luxury to expect.

What, in these circumstances, might be done? The best chance is that the foundation may be able to move on from identifying barriers to mobility within Europe to becoming a monitor of what is happening within European science. There are housekeeping jobs to do, such as the compilation of the up-to-date register of who does what research, now done by some European governments, but always unsystematically. There is also the more contentious task to tackle of inducing among the European research councils (most of whom are members of the foundation) a sense that coordination of some kind would be desirable. Even a device as simple as trying to produce an annual report on European academic science would be a sensible starting point. But these are only beginnings. □

Italian research

New president's ambitions firm and unclouded

Rome

PROFESSOR Luigi Rossi Bernardi, the respected haemoglobin physiologist who is the new president of the Italian national research council (CNR), is moving like a whirlwind through the dusty corners of this dominant institution of publicly-funded science in Italy. But some old hands are already wondering whether the wind will really clean up the moribund organization or merely raise dust.

Whatever the outcome, the new president is starting as he means to go on, not least by introducing real (if presently crude) measures of output and scientific quality into the CNR system, where many groups and projects, critics say, are continued long beyond their useful life. Thus, within days of his appointment, Rossi Bernardi obtained tapes of world publications and citations from the Institute for Scientific Information in Philadelphia, and began analysing them on a computer in Milan for the publications of CNR groups and institutes.

The analysis took a week of computer time, but threw up results such as that some ten CNR groups or institutes (out of 270) had published nothing for five years. Such groups will be closed, Rossi Bernardi promises.

And at an extraordinary meeting last week in Rome, nominally to present the annual report to the assembly of CNR which was in practice the new president's first chance to present his policies publicly, these policies were alternatively vigorously supported, barely criticized and finally approved. According to one enthusiast, who spends half his time at the US National Institutes of Health, the occasion was fundamental in that it represented a thorough changing of the old guard.

As if to emphasize the political nature and certainly the importance of the task ahead, Rossi Bernardi was flanked throughout last week's meeting by the minister of research, Mr Luigi Granelli, who seems at least as determined as his new CNR president to change the face of Italian science. It was the first time in the memory of some observers that a minister had spent so long at a CNR meeting — which ran into a second day, with a seminar on the international relations of the council. In that half of the meeting, distinguished Italian scientists working abroad presented their comparisons of foreign (often American) procedures with those in Italy, thus — inevitably — lending weight to Rossi Bernardi's own proposals. Italian science attachés from Washington, Moscow and elsewhere were also afforded their own time for criticisms. Rossi

Bernardi later made some "quite exciting proposals", according to one attaché, for the revitalization of the attaché network — thus emphasizing the importance the new CNR president attaches to avoidance of parochialism.

Granelli also pledged himself to removing the "parastatale" status of the CNR, which equates CNR scientists with the meanest of Italian civil servants, and whose effect is to make promotion entirely dependent on age. He also estimated present Italian research and development spending as 1.3 per cent of gross national product (it was under 1 per cent at the end of the 1970s), and set a target of 2.5 per cent by the end of the 1980s. There was a 40 per cent increase in public spending on science in Italy last year, it was claimed.

Rossi Bernardi's objectives for 1985 include:

- Decentralization.

- Cutting a tangle of red tape and obscurity in CNR financial management, including a new separation of administrative and research expenditure.

- Setting up a databank on CNR personnel.

- Monitoring scientific output.

- Better control of the "finalized projects" (through which, in practice, many university groups raise funds for only nominally applied research projects).

According to the minister of research, such reform is necessary not only for CNR, but should act in CNR as a "nucleus" for the reform of science in all Italy.

All stirring stuff — but some sceptics in Rome last week felt the momentum would finally dissipate in the treacle of Italian bureaucracy, while others felt Rossi Bernardi to be well-meaning but politically naive in a country where politics is all. Granelli, who is strongly behind the new CNR president's initiatives, may also not last long — few Italian ministers do, as governments fall frequently, or he may be promoted — when Rossi Bernardi may find himself exposed and rather alone. But as Granelli himself said last week, Italy faces "marginalization" if research is not taken in hand.

Robert Walgate

NIH research

Reducing grant-writing burden

Washington

THE US National Institutes of Health (NIH) are to consider extending the durations of their extramural research project grants in order to reduce the burden of preparing grant applications and to provide reviewers with better evidence on researchers' performance. This general proposal was well received at a meeting last week of the NIH director's advisory group, and NIH director James Wyngaarden said after the meeting that he took the favourable response as an "endorsement in principle" for extending grants to first-time applicants beyond the usual 3 years. Grants to mid-career and distinguished investigators might also be extended.

The move comes in response to growing concern in the academic community that the pressures created by the system as at present operated — termed "intimidating" by Wyngaarden — are impeding research creativity. Most first-time grant holders must start preparing renewal applications after less than 18 months' work — hardly enough time for a realistic appraisal of progress. Furthermore, researchers often spend up to three months drafting applications, because most believe, probably correctly, that their chances of a renewal depend on the result of a detailed scrutiny of the proposal.

Several invited participants from academic institutions last week urged a return to the philosophy of investment in promising scientists, rather than "procurement research". There was dissatisfaction that

"study groups", the first level of peer review for grant applications, spend too much effort examining minutiae and "micro-managing" projects. Many suggested that reviewers would be able to make more informed judgements if most first-time grants were to be extended to, say, five years. One proposal that Wyngaarden found attractive was that grants might specify a fixed amount of money that could then be spent over a longer or shorter period depending on progress. This solution might allay the anxiety of having to produce results within three years, while avoiding substantial extra commitments on the NIH budget. However, no formal vote was taken, and Wyngaarden stresses that any change to present practice will first be subject to internal review.

Dr Joshua Lederberg, president of Rockefeller University and a leading critic of present practice, said it was not yet clear how any change should be implemented, and stressed the need for more information on the careers of those who fail to obtain grant renewals and disappear from NIH records. Academic institutions are becoming less able to find alternative work for such people, and the average duration of tenure of a trained researcher within the extramural system is only seven years, which Lederberg thinks represents a wasted investment. Reviews emphasizing the track record of the applicant would, he feels, bring much-needed stability to the careers of mid-term investigators.

Tim Beardsley

European air pollution

Commission's directive in balance

Brussels

UNLESS they can hammer out several major political compromises, environment ministers of the European Communities meeting here next week (6 December) have little chance of getting to grips with the problem of air pollution.

For the past several weeks, discussions of the draft directive on limiting emissions from large combustion plants have shown little sign that the member governments are prepared to relax the positions they defended at the last council in June. If anything, they seem more firmly entrenched, at least to judge from the number of objections lodged to this important and controversial piece of Community legislation.

The Commission wants to see air pollution programmes in force by 1 January 1985. The draft directive would set uniform emission standards for sulphur dioxide, nitrogen oxides and dust from plants with a thermal output of 50 megawatts or more. Its aim is to reduce, by 1995, the volume of sulphur dioxide emitted by 60 per cent (compared with 1980), and dust and nitrogen oxides by 40 per cent.

The member governments, however, remain divided both on the particular emission limits in the draft directive and on the principle whether there should be global emission standards at all.

In the forefront of opposition to the directive is the United Kingdom, which argues that deadlines are not necessary because scientific evidence on the contribution of industrial plants to air pollution is still incomplete, and that the cost to industry of implementing the directive would not be offset by its effects on the environment. According to the Commission, the extra cost of fitting new combustion plants with desulphurization equipment to remove 90 per cent of sulphur from fuel gases to achieve the limit of 400 mg of sulphur dioxide per cubic metre would be US\$100 per kilowatt of electricity produced.

Other opponents of the directive are Greece and Ireland, both of them conscious that their industrial emissions are likely to increase in future. Greece is planning new generating plants using its own indigenous and particularly polluting fuel. Ireland, for this half-year the president of the council, is in a ticklish position, having to show itself in a forceful role while having to replace much natural gas usage by coal-fired electricity generating plants.

Luxembourg, which has no plants that would now be affected by the directive, is also worried about the future, while Italy, which also opposes the directive, is asking that emission limits should be fixed nationally, not at Community level.

The emission limits proposed by the Commission are, however, considered too

weak by West Germany and the Netherlands. Denmark, which is in favour of the directive, also considers the emission limits too strict, especially for nitrogen oxides and dust.

Indeed, several governments would prefer to see dust left out of the directive altogether, partly because monitoring of dust emissions can be up to 50 per cent in error and because of the lack of data about present practice. The British want the directive to apply only to steam or electricity generators and not to the cement works, brick works and not/or treatment plants that would at present be included. While some (including France, Ireland, the Netherlands and Italy) consider these a dangerous source of nitrogen oxides, other governments insist that control technology is at present inadequate for the directive to be effective.

There is also disagreement about the size of plants to which controls should apply. The United Kingdom has proposed a 100 MW threshold, Ireland 150 MW. Although the Commission's proposal includes a number of temporary exemptions, as when costs of compliance would be disproportionately high or when total annual emissions are lower than in other countries, even these are considered insufficient by countries such as Greece, Ireland and Luxembourg.

The Irish presidency's search for a compromise is turning out to be difficult. Three proposals have been put forward.

- Limiting the directive to new plant, which West Germany fears would allow too much plant now planned or being built to escape regulation by the time the Community directive is turned into national law.

- Raising the threshold to 150 MW, to which some object that the result would be more small plants and thus more pollution in total.

- A quota system with national emission rates fixed on criteria such as the level of energy consumption and the contribution to transboundary pollution. Belgium objects on the grounds that this compromise would be discriminatory and West Germany on the grounds that it violates the polluter-pays principle. The Commission itself fears that this compromise would conflict with Community law.

At next week's meeting, the ten governments will be equally divided, with Britain, Greece, Ireland, Italy and Luxembourg in opposition. A further complication is that the Commission's draft directive has been severely criticized by the European Parliament on the grounds that it is too lax. Ursula Schleicher, West German Member of the European Parliament, for example, says that the five-year exemption for plants below 100 MW will generate the building of small plants. She also advocates a

supra-regional monitoring system for air pollution and financial support from the Community to deal with regional problems.

The European Parliament itself has called for the application of the standards to existing plants and for stricter emission values by the end of 1990.

Another contentious issue next week is that of phasing lead out of petrol (see *Nature* 4 October, p.401). The council will be able to take a decision only in principle, because the European Parliament's opinion has been blocked by the 158 amendments submitted during its last session.

Denmark, Luxembourg and the Netherlands have, however, joined West Germany in their willingness to introduce lead-free petrol by 1986 while others would agree that this date should be optional (with the exception of Greece, which is unhappy about the whole thing). France is saying that the interim standard of 0.15 grams of lead per litre of petrol need not be included in the directive.

Among the other issues that will be discussed next week are a proposal that Community governments should aim at US emission levels from automobiles by 1986 rather than 1988, but again there are differing opinions about the need for an interim set of emission standards. The meeting will also have to decide whether to spend 100 million ECUs on a five-year programme to protect forests from fires as well as acid rain, chiefly by setting up a monitoring network.

Anna Lubinska

New climate unit

A NEW centre for atmospheric and oceanographic research was opened at the University of Oxford two weeks ago by the chairman of the UK University Grants Committee, Sir Peter Swinnerton-Dyer, in his capacity as chairman of the Meteorological Committee, which answers to the Secretary of State for Defence. The new centre, which promises to play a significant role in both satellite meteorology and large-scale oceanographic-climate modelling, is unique in being administered and supported by the Meteorological Office while being located in a university department — Oxford's department of atmospheric physics.

Most of the staff at the new unit are drawn from the Meteorological Office, with the addition of Dr Adrian Gill, a theoretical oceanographer (who until recently was at the University of Cambridge). The staff are drawn equally from the fields of numerical oceanography and satellite meteorology, and the expectation is that close proximity to the atmospheric and remote sensing expertise at Oxford will focus and stimulate research on such problems as tropical climate-ocean interactions and satellite sea-surface measurements.

Philip Campbell

Gene therapy

US clinical trials imminent

Washington

THE way is now being cleared for the first human gene therapy experiments, which could take place as early as next spring.

One research group, headed by Dr Ted Friedmann of the University of California at San Diego, has already received permission from the local Institutional Review Board (IRB) — the panel charged with protecting human research subjects — for clinical trials of a procedure aimed at correcting the genetic defect responsible for Lesch-Nyhan disease, although the proposal would still have to be approved by the National Institutes of Health Recombinant DNA Advisory Committee (RAC) before trials could begin.

In anticipation of Friedmann's and other proposals, RAC earlier this year established a working group on human gene therapy which is in the process of drawing up guidelines that will tell researchers what information to submit with their proposals. The working group, which includes ethicists as well as physicians and researchers, will also screen all such proposals before presenting them to the full RAC for consideration.

A draft of the guidelines, known as *Points to consider in the design and submission of human gene therapy protocols*, makes it clear that RAC plans to look beyond the questions of risk and protection of human subjects in its scrutiny of proposals. Many members of the working group strongly favour expanding RAC's purview to include such social issues as whether the procedures will be subject to proprietary rights and whether the proposed somatic-cell treatment could affect the reproductive cells of the patient.

Nevertheless, it is considered unlikely that the working group or the full RAC will turn down legitimate proposals from researchers seeking to perform somatic-cell gene therapy. One scientific member of the working group has in fact resigned in impatience at needless hand-wringing over a foregone conclusion.

Meanwhile, a broad consensus appears to have emerged among religious leaders and ethicists that although germ-cell therapy — that is, alteration of heritable traits — raises difficult ethical problems, no such objections exist in the case of somatic-cell therapy. The Office of Technology Assessment (OTA), which is releasing a study on human gene therapy next month, found strong support for this view among a group of theologians, religious leaders and ethicists it assembled for a meeting in September.

The Food and Drug Administration (FDA) is also preparing itself for the first gene therapy experiments. In an announcement to be published shortly in the *Federal Register* (and which will be part of a larger statement of overall federal policy on the

regulation of biotechnology being coordinated by the Office of Science and Technology Policy), FDA will make clear that it intends to view recombinant DNA used for human gene therapy to be "biologicals" subject to FDA regulation.

Before beginning clinical trials, researchers would have to apply to FDA for an "investigational new drug" permit for the recombinant DNA; and FDA would have to approve the product's safety and effectiveness before its general use could begin. (Unlike the RAC guidelines, which are technically binding only upon research institutions that receive federal funds for recombinant DNA research, the FDA procedures have the force of law.)

The OTA report notes four research groups that are close to clinical trials. According to NIH officials, at least one

grant application recently received by the genetics study section includes a proposal for human trials. All three of the target diseases mentioned in the OTA report as likely early candidates involve single gene defects that result in enzyme deficiencies.

One candidate is Lesch-Nyhan disease, recognized by renal failure and central nervous system disorders. The others are purine nucleoside phosphorylase deficiency and adenosine deaminase deficiency, both recognizable by the severe immunodeficiency they cause. Stuart Orkin (Harvard University) and David Martin (University of California, San Francisco, in collaboration with Genentech) have set their sights on genetic replacement of the deficient enzymes.

In each case, the defect is in the bone marrow; the most likely clinical approach would be to remove the patient's marrow, infect the cells with "repair" DNA carried by a retro-virus, and then replace the marrow.

Stephen Budiansky

Japanese telecommunications

Future goes up in flames

Tokyo

A LITTLE forgetfulness on the part of a Tokyo telephone repair man last week almost certainly caused Japan's first "telecommunications disaster" — and a heated debate about the vulnerability of the new "electronic society" into which the nation is rushing headlong.

What the repair man apparently forgot to do was to remove a small piece of rag wrapped around telephone cables in a lead pipe he was soldering. The result was a fire that spread into underground ducting and destroyed 90,000 telephone, databank and telex links, cutting off telecommunications in one whole ward of Tokyo and bringing a large part of the nation's banking operations to a halt.

Worst hit was Mitsubishi Bank, the nation's fourth largest. The fire cut the vital link between its central mainframe computer, which holds details of 6 million customers' accounts and records 200,000 transactions a day, and the office that relays the data to its 217 branches. All Mitsubishi's banking operations throughout Japan immediately came to a complete halt. Three more of the nation's top five banks also lost links with central computers and had to stop transactions in and around Tokyo, and 48 post office branches close to the fire had to halt savings account operations.

Businesses in the area were thrown into confusion. One of the big security companies had to stop trading when its computer was cut off, a major parcel company lost access to the central computer that records the routing and delivery of the 600,000 packages it handles each day, hospitals lost access to medical records normally sent by facsimile, the major overseas news wire service lost contact with

foreign offices, and hundreds of retail and wholesale businesses saw telex, credit card and telephone orders vanish and supermarkets unable to order fresh stock.

Residents of Settagaya ward, where the fire broke out, slipped back into the pre-telephone era. All telephone lines were cut, including those for emergency services, so that people had to report crimes and fires to local stations on foot. To try to see things through, the telecommunications monopoly, NTT, brought in armies of couriers to relay messages between the central telephone office and local residents — four days after the fire, nearly 5,000 messages a day were being carried. A satellite link was provided from a mobile transmitter, a mobile exchange brought in and 800 public telephones set up in the street — some 30 minutes of queuing was, however, needed to make a call.

Frantic efforts by NTT to restore telecommunication services have continued, with 10,000 man-days of work in the week since the fire. Telephone lines should soon be fully restored, although it will be another couple of weeks before all the damage is repaired.

Meanwhile, businesses in the affected area are finding to their chagrin that provisions for compensation are hopelessly out of date. An appeal to NTT by 600 shop owners on the grounds that "telephone paralysis is a new kind of city disaster" is not likely to meet with a generous response. The public telecommunications law, adopted around 30 years ago, provides only that if a telephone service is stopped for more than five days, compensation will be provided at the same rate as the basic rental charge; that means that compensation would reach about £20 after a three-week stoppage.

Alun Anderson

Informatics

Olivetti bids to rival IBM

Milan

OLIVETTI, the Italian-based typewriter and office furniture company now well-launched into a transformation into an informatics business, aims to become Europe's IBM, said economic studies director Bruno Lamborghini at a meeting at Olivetti's headquarters at Ivrea last week. But the company has a long way to go. It ranks tenth among world informatics technology companies, and is second in Europe only if it includes sales of electronic typewriters. Moreover, first place in Europe is taken by IBM itself, with seven times Olivetti's European sales. But Olivetti is chasing fast: its consolidated profits in 1983 were triple those of 1982, and its sales volume curve is at present rising like the graphs normally seen only in cartoons.

The rub for Europeans, however, is that Olivetti's rise and rise is only partly a European phenomenon. Although nobody would say so explicitly, it is clear that Olivetti has written off any major prospect of growth by combination with other European partners. Instead, it is relying on the deal made earlier this year with a big wolf, the American Telephone & Telegraph Company (AT&T, formerly Bell Telephone), which has injected capital into Olivetti to a total now reaching a quarter of the outstanding equity.

The deal allows the fraction ultimately to reach 40 per cent, coming close to control, but in a display of bravura occasioned by Olivetti's tremendous success lately under its dynamic president, Carlo de Benedetti, the company shows no fear that it will act merely as the back door by which the American wolf will enter Europe.

Indeed there is talk in Ivrea of showing AT&T how to make proper use of its wasting asset, Bell Laboratories, which are seen as "good for Nobel prizes" but relatively weak on applications technology for the new informatics market. Olivetti now has a hundred or so people at Bell Labs at any one time, where they have access to all but defence and public switching work. This offers Olivetti "a unique opportunity", says Lamborghini. The company has always been conscious of design, which in turn means marketing, which in turn means the needs of office users — which is where the information revolution is taking place. The combination of Bell research (and communications technologies) and Olivetti's closeness to the office could be a very powerful combination that would leave mainframe dinosaurs such like IBM behind. Such is the stuff of dreams at Ivrea.

But what of combination in Europe? "The political and economic discord that currently divides the European countries does not permit optimism on possible progress in the immediate future", write

Lamborghini and the corporate marketing director Vittorio Cassoni cautiously in a joint report. Esprit, the joint European project on fifth generation ("intelligent") computers, is welcomed at Olivetti but seen as "very small" compared with the efforts of US manufacturers, said Lamborghini last week.

It is playing an important role in the definition of common European standards, however. Esprit provides a point of contact where companies can meet ("it was incredible how the European companies, even in the same country, were isolated") but there is frustration in Olivetti at how national and inter-company rivalries are hindering the project. Thus Olivetti directors, asked whether the company has decided to go it alone (with AT&T) without the help of Esprit, shrug their shoulders in a gesture of "what do you expect?", and smile.

European Commission

Golden parachutes at Ispra

SOME 120 high-grade scientists and technicians earning between £25,000 and £40,000 a year at the European Commission's Joint Research Centre (JRC) at Ispra, in Italy, are to be offered early retirement on terms starting at 70 per cent of their current salary. Or so says a proposal now before the European Parliament for its "opinion". According to Glyn Ford, Labour Member of the European Parliament and once science policy lecturer at the University of Manchester, that opinion should be "no" — although the Energy, Research and Technology Committee to which he belongs has voted the measure through.

Ford says the objective of the proposal is "perfectly sensible", for it is aimed at shifting 50–60-year-old nuclear physicists who were given lifetime appointments when JRC began as a Euratom research centre 25 years ago, and bringing in younger, more junior scientists in new disciplines. The European Parliament and the Commission have both been urging JRC to rejuvenate the shift to non-nuclear technologies and environmental issues, and the retirement scheme is a response to that.

"But I object to the quantity of cash involved and the method by which people are being retired", said Ford on Monday. The retirement scheme will be voluntary and unrestricted, "so only the best people will go". There has long been doubt about the scientific productivity of JRC, and this could make the situation worse, Ford believes. The result of a similar scheme at Manchester, Ford says, meant that all the people who could get jobs, and were in demand (such as computer scientists and accountants) left, depleting the university

As for research directions, Olivetti is interested in Bell Labs' flat screen (plasma) technology, in non-impact (thermal and ink-jet) printing, and above all in voice input and output. Artificial intelligence (AI) is also seen as "crucial and strategic", requiring research at many levels and in many directions, as intermediate products — particularly involving voice recognition — will be affected by intermediate developments in AI research. But Olivetti does not intend doing basic research in AI — it will leave that to Bell Labs.

As for products, Olivetti research director Arnaldo Pasini foresees a laser printer with all the graphic capabilities of a newspaper, a digital reader (to record incoming letters etc. in bit image form, so there will be no need to keep paper files), and vocal input and output, all within a few years. Most innovative technology will come from the United States, where Olivetti has now invested in some 20 venture companies, Pasini says. Olivetti's job will be to match it to the market: to become, says one commentator, "the great transformer".

Robert Walgate

of a useful cadre of people. Moreover, although those taking retirement from JRC will be obliged to notify the centre if they subsequently begin earning enough to take their total income (including pension) beyond their original income, the scientists come from many different countries and violations will be difficult to trace. The result will probably be a brain-drain "to our competitors" financed by the European Community, Ford claims.

JRC director Jean-Albert Dinkespieler, however, puts the matter in a slightly different perspective. The net cost to JRC will be 1.5–2 million ECU (about £825,000–£1,100,000) a year for ten years, only 1–2 per cent of the JRC salary bill, when account is taken that money will be saved by replacing highly paid by younger less well-paid people. Moreover, he points out, all new appointments will be on a contract (5-year renewable) basis, to avoid a repetition of the problem now being faced. Similar schemes have operated successfully at the European Space Agency, he claims. The researchers were too old to change speciality, and too young to retire, so what else should be done to create movement?

It would probably be a one-off exercise, said Dinkespieler, and would involve recruitment of biologists (an innovation for Ispra), air and water chemists and oceanographers for pollution studies. The Council of Research Ministers has given its outline approval to the scheme, he said, but European parliamentary approval (which involves the advice of many committees including Energy, Research and Technology) was also necessary. Ford's intervention had made matters more difficult.

Robert Walgate

Australian nuclear policy

Non-nuclear election party

Canberra

THE Australian Labor government's uranium export policies and the combined presence of joint US-Australian defence facilities at North-West Cape, Nurrungar and Pine Gap are looming large as issues in the forth-coming federal elections to be held on 1 December. Perhaps the most unexpected development in the run-up to the election has been the swift rise to prominence of a new single-issue political party — the Nuclear Disarmament Party (NDP), formed only in June, which appears likely to capture at least one seat in the half-Senate election, if recent polls are to be believed. The polls also suggest that the government will retain power in the lower house, with the personal popularity of the Prime Minister, Mr Bob Hawke, a large factor.

The Senate (the upper house), however, is the Achilles heel of the Labor Party, and triggered the downfall of the Whitlam government in 1975. In a Senate in which the government may again not gain control, NDP says that if elected, its members will vote only on questions relating to uranium, disarmament and the joint facilities. Ironically, this would limit their capacity for log-rolling deals, since the conservative opposition parties are likely to vote with the government on most nuclear issues. Mr Hawke said that a vote for NDP would be wasted and hastened to point out the Labor government's achievements in the disarmaments sphere. He added that, as the South-East Asia/Pacific region member country "most advanced in the technology of atomic energy including the production of source material", Australia has a designated seat on the board of the International Atomic Energy Agency and has recently become a member of the United Nations Security Council.

Since July 1983, Australia has been represented in international forums by an ambassador for disarmament, Mr Richard Butler, and funds have been made available for a peace studies centre at the Australian National University, in support of the comprehensive test-ban. As well as attempting to help solve the procedural problems that have plagued the conference on disarmament, Australia is also playing a large role in the current technical trials of an international test-ban verification network, run by the Geneva Group of Scientific Experts on Seismic Events (GSE). Coordinated from the Bureau of Mineral Resources in Canberra, Australian seismic stations at Alice Springs (Northern Territory), Narrogin (Western Australia), Charters Towers (Queensland) and Mawson (Antarctica) transmit data to Melbourne for relay to the other centres (Washington, Moscow and Stockholm) through the World Weather Watch telex system.

But the disillusioned ex-Labor supporters and young people of NDP reject the government's whole nuclear package. They believe Australia's best interests lie in closing the joint defence facilities and in leaving Australia's large reserves of uranium in the ground.

Jeffrey Sellar

European all-change

PROFESSOR Eugen Seibold, president of the *Deutsche Forschungsgemeinschaft*, was appointed president of the European Science Foundation at the tenth annual meeting of the foundation in Strasbourg last week. Professor Seibold succeeds M. Hubert Curien, president of the foundation since 1980, who has felt it necessary to resign on his appointment as Minister of Research in the government of France. Professor Seibold has been active in the foundation's activities since its inception, recently as vice-president.

The Strasbourg meeting last week also appointed as the foundation's secretary-general Mr Michael Posner, until two years ago chairman of the British Social Science Research Council. Mr Posner succeeds Dr John Goormaghtigh, secretary-general at Strasbourg for the past six years.

The members of the foundation last week agreed that the budget for the coming year should amount to just under FF10 million, an increase of some 9 per cent compared with the previous year. In addition, the members of the foundation, mostly grant-making research councils, have agreed to contribute an equal amount to the cost of the foundation's "additional activities" — cooperative research projects to which interested members contribute and in which they usually participate. □

Acid outback rain

Canberra

ACID rain in the outback of Australia? Surely not. Yet this is the conclusion of two independent teams of atmospheric scientists on the hydrogen-ion concentrations in rainwater at sites hundreds of kilometres apart in Australia's Northern Territory. The teams, led by Dr Gene Likens of the Institute of Ecosystem Studies of the New York Botanical Gardens and Dr Barry Noller of the Office of the Supervising Scientist, Alligator Rivers Region Research Institute, presented their results at an international conference on the scientific application of baseline observations of atmospheric composition, held earlier this month at the Commonwealth Scientific and Industrial Research Organization (CSIRO) Division of Atmospheric Physics in Ascendale, Victoria.

Both groups found that at their respective rainfall sites at Katherine, 200 km

Yugoslavia

Brain drain

AN "irresponsible attitude" towards science and scientists has produced a massive brain drain from Yugoslavia, according to a new survey from the Zagreb Centre for Migration Research, reported by the Belgrade daily *Politika-Ekspress*.

According to the latest figures, during the past two decades some 5,000 Yugoslav specialists have left, with a peak of 1,371 leaving in 1968-73. The basic reasons, according to *Politika-Ekspress*, are "bureaucratic harassment", inadequate opportunities for promotion, or even the total lack of a job, and the desire for higher earnings and a higher standard of living. Vujica Jevdrevic, an expert in hydrology employed by the United Nations, who could not obtain "adequate conditions to continue his work" in Yugoslavia, and decided to remain in the United States. The "celebrated mathematician". The nuclear physicist Bogdan Maglic was fired from his Yugoslav post as a result of a bureaucratic error, while he was in the United States doing advanced studies, so that he decided not to return.

According to *Politika-Ekspress*, the brain drain is motivated by practical and economic considerations, and there are (implicitly) no political factors involved. Indeed, it is noted that "the highest political bodies and the Yugoslav leadership" have recently been demanding that the "irresponsible" stance of the bureaucrats towards science and scientists should be substantially reformed. It is noteworthy, however, that the peak years of emigration, 1968-73, cover the purge of scholars associated with the Matica-Hrvacka movement in Croatia, and a subsequent similar purge of intellectuals in Serbia.

Vera Rich

south-east of Darwin, and Jabiru, 300 km east of Darwin, the average hydrogen-ion concentration was about one-third that of the corrosive acid rains of northern Europe and North America. The difference is that the sulphate and nitrate ion concentrations are at least an order of magnitude less than the Northern Hemisphere measurements and that the high hydrogen-ion concentration is accounted for by the unexplained presence of formic, acetic and other weak acids. At some sites, pH readings as low as 3 have been recorded. The organic acid concentration is highest in the early part of the wet season (November-December) and lowest in the monsoon period (January-March). Dr Likens is reported to have been keen to undertake the 30-month study in order to determine what atmospheric chemistry was like "before the Industrial Revolution".

Jeffrey Sellar

Creationism in confusion?

SIR — It was, perhaps, too much to expect the founders of the "Association for the Protection of Evolution" (APE) to give an objective report on the recent Biblical Creation Society (BCS) conference at High Leigh (News and Views, 25 October, p. 703). What is surprising, however, is that you should give such extensive and uncritical exposure to views which were self-evidently biased.

The very heading to your report, "Creationism in confusion", betrays a tendentious purpose, namely to present BCS in the worst possible light. Every difference of opinion or continuing debate among creationists is represented as evidence of "confusion" rather than the product of honest enquiry and developing ideas. By the same token, would the APE-men agree that evolutionary theory is "in confusion" because neo-darwinians, punctuated equilibriaists and reformed cladists disagree? Debate and disagreement is the stuff of scientific and intellectual progress and the BCS open day demonstrated that (contrary to the impression given) creationists are capable of constructive self-criticism at the highest scientific and theological level.

The conference report contains several serious errors of understanding. Howgate and Lewis fail to grasp the purpose of David Tyler's lecture which was not to reinterpret geology in terms of catastrophism but to illustrate from field observations (1) that the straitjacket of Lyellian uniformitarianism is a hindrance to the science of geology and (2) that there is a need to test the appropriateness of modern analogues, whether catastrophic or non-catastrophic.

Dr Darnborough, described as "BCS's top geneticist" is, in fact, a molecular biologist. He does not "believe in evolution". His paper argued, rather, that the operation of accepted evolutionary processes at the molecular level upon genetically perfect *created* organisms would lead to a living world with genetic characteristics essentially similar to those observed in modern organisms. This paradigm is fully consistent with both the scientifically observed facts and the biblical doctrines of creation and the fall. It further avoids the need to invoke the vast improbabilities of chemical evolution.

My own lecture "Christ and the Cosmos" is represented as a "keynote theological policy statement" and a condemnation of "American creationists". It was nothing of the kind. It was a positive exposition of an important New Testament passage which sets out the relationship between Christ and the created, divorcing "creationism" from the Creator, and illustrated this by reference to certain trends in American creationism. To suggest that I "condemned" or "attacked" the whole US creationist movement is so far from the truth that I am tempted to attribute to your reporters the unworthy motive of using

your journal to sow international discord among creationists.

The report continues with the statement that "a stalwart of the rival creationist organization the Creation Science Movement attacked the backsliding into theism of the BCS". First, there is no rivalry between BCS and CSM. The two societies have somewhat different aims but remain in close and amicable consultation. Second, your correspondents should learn a few basic theological terms before venturing into print on such subjects. Both BCS and CSM are proud to be "theists". It is "deism" that we, equally, disown.

Finally, we are astonished that you should include, as part of the conference report, a private conversation over tea between one of the APE representatives and Dr David Gower. The ethical lapse is the more serious since Dr Gower is seriously misrepresented as admitting "unenthusiastically" that he ascribed genetic defects to the fall and that sexual communication by pheromones has to do with "lustful temptations". These serious misrepresentations of a senior university academic are, to say the least, deplorable.

What your correspondents fail to grasp is that creationism is an holistic philosophy which seeks to understand the scientific enterprise within a biblical and theological framework. In pursuing this concept, and rejecting the arid materialism which underlies so much modern thinking, we are simply returning to the paradigm espoused by such as Newton, Boyle, Kelvin, Faraday and Maxwell, who among others laid the foundations of science as we know it.

E.H. ANDREWS

Department of Materials,
Queen Mary College,
Mile End Road, London E1 4NS, UK

Riposte on CERN

SIR — Victor F. Weisskopf and J. H. Mulvey (*Nature* 8 October, pp. 599-600) make various criticisms of our study of CERN, the European laboratory for particle physics. The criticisms are unfortunately misplaced since they relate to a brief *Nature* article (6 September, p. 4) rather than to the detailed findings recently published in two parts in *Research Policy*.

First, we do not base our "main conclusions on a selection of so-called 'crucial' experiments" — we actually devote as much attention to evaluating "incremental" contributions to science. Indeed, the very first conclusion listed in the second of our papers is that since 1970, "the overall record of the CERN machines taken together has been better than that of the accelerators at any other laboratory in the world when judged in terms of experiments producing precise measurements". Nowhere do we describe such work as "dull physics".

Second, Mulvey's assertion that there is no evidence to support our conclusion that the multinational nature of CERN has previously led to problems in research management is wholly inaccurate. Even the most cursory reading of our papers would reveal that our evidence comes from interviews with more than 180 high-energy physicists, many of whom cited this particular problem as important.

Third, it is gross oversimplification by Mulvey to suggest that the greater success of the United States in terms of important discoveries was due to a single machine. The Brookhaven Alternating Gradient Synchrotron had a far better record than the very similar CERN Proton Synchrotron, as did Fermilab compared with the CERN Super Proton Synchrotron (SPS), while the CERN Intersecting Storage Rings "missed" several major discoveries in their energy range.

Fourth, the successes of Stanford with the SPEAR collider were not entirely unpredictable. Among other factors, they can be attributed partly to the fact that the machine opened up a new energy range (unlike the CERN SPS which began operating four years after an identical energy US machine), and partly to its users, who already boasted an impressive track record in exploiting the earlier Stanford linear accelerator.

Lastly, one of our papers devotes considerable attention to identifying the factors that have structured the performance of CERN accelerators. It is therefore incorrect to say that we offer no explanation for the improvement over recent years.

BEN MARTIN

JOHN IRVINE

Science Policy Research Unit,
University of Sussex,
Falmer, Brighton, Sussex BN1 9RF, UK

Irish students

SIR — We were surprised by your statement (18 October p. 592) under the heading "Irish disunity" to the effect that "Roman Catholic students from Ulster tend to seek higher education south of the border when (*sic*) they are welcomed as if Irish".

This statement is patently false and could be unwittingly damaging. Although the Queen's University of Belfast, being completely non-sectarian, does not keep statistics of students' religions, it is common knowledge that the proportion of Catholic students at Queen's easily exceeds the proportion of Catholics in the population of Northern Ireland as a whole. The several thousands of Catholic students at Queen's alone, apart altogether from those in the non-sectarian University of Ulster, constitute a group at least four times larger than the total number of all Northern Ireland students who attend universities in the Irish Republic.

I.D. STRAHAN

The Queen's University of Belfast,
Belfast BT7 1NN, UK

Mind, body and intelligence

This year's BBC Reith lectures are arresting and provocative but also representative of the kind of argument that gives philosophers a poor reputation in research laboratories.

CAN machines think? And if so, in what sense? Those are two of the simple questions with which Professor John Searle of the University of California has been starting the British Broadcasting Corporation (BBC)'s audiences in the past few weeks. Professor Searle is this year's Reith lecturer, which means that he is one of a long line of distinguished scholars licensed to talk at British audiences for half an hour on each of six consecutive weeks. To judge from the first four of these occasions, Professor Searle is an iconoclast in the literal sense, one concerned with the destruction of idols. And there is no reason to think the worse of him on that account. But on this occasion he may have chosen too many idols, in too breathless a rush of broadcasting time, to satisfy the intellectual curiosity of his listeners.

Searle's general title is arresting — *Minds, brains and science*. His announced objective is to explore the relationship between the concepts of mind and body as illuminated by what has been learned of neurophysiology in recent years with the particular intention of trying to make sense of concepts such as free will, or "intentionality" as he puts it. And certainly it is an important question to answer, how to reconcile the behaviourists' view of people as predetermined exemplars of the laws of mechanics with the illusion we all share that in some sense we are in charge of our own destiny, or of events around us. Or, more accurately, where is the line to be drawn between the domains of conditioned and voluntary behaviour? And how fuzzy is that line?

We shall have to wait to see how Searle comes out on these questions. One striking measure of his success so far, however, is that he has made some of the subsidiary questions with which he has dealt into cocktail-party conversation. Is it, for example, true, as Searle asserted in the first of his lectures, that there is no serious problem about the distinction between mind and body? Body means a collection of neurones and mind means the qualities of consciousness, intentionality and so on that we attribute to ourselves. Searle's answer is that neurones are to be likened to the atoms that constitute physical objects, and that mind is the consequence of putting them together as they are in people, in every way the analogue of the external shape of the object concerned.

All this is unexceptional, even if it does not say much about the nature of the attri-

butes of the macroscopic representation, the nature of consciousness, for example. No doubt even philosophers will think Searle's answer a little over-simple, but it is no less arresting on that account. The circumstance that it leaves the question of how consciousness springs from the human nervous system to be answered by the neurophysiologists is neither surprising nor unwelcome, for the question is interesting and important.

The more serious difficulty with Searle's iconoclasm so far is that he seems to have chosen for attack some idols that are conspicuous by being insubstantial, which is where the question arises of whether machines can think. In a nutshell, Searle has been tempted into the sport of making fun of the community of computer-people who believe their creations are capable of artificial intelligence, or AI as they call it. As everybody knows, it is an easy sport, like shooting sitting ducks. It is also a misguided sport, certain with a British audience to evoke smug declarations that it has been clear all along that artificial intelligence is unreal.

Searle has made his point by means of a memorable example, one much talked about as irrefutable. Put a man in an empty room but provide him with a set of rules for combining Chinese ideograms together in ways that make sense (to Chinese speakers). Then imagine that a number of Chinese speakers outside the room are able to enter bundles of ideograms which the man must combine together according to the rules provided. To those outside the room, the man may well (when he is well practised at his task) be performing like an intelligent machine. But to the man, the process will be devoid of meaning. In this setting, Searle says, the system of man/room is exactly equivalent to a digital computer. If the people outside have omitted some essential rules, the system will be an unintelligent computer system.

The analogy is good because it is arresting and because it is also susceptible to further analysis. The difference between the man in the Chinese room and an intelligent human being, Searle says, is that the former has been provided merely with the formal syntax of the language with which he is working — the rules for telling how symbols should be combined — but that human beings have learned to attach meaning to the symbols. Semantics are an essential part of the intelligent manipulation of language.

Careful listeners to what Searle has been saying will recognize some important qualifications of what appears to be his case, that machines cannot think in the true sense of the word. First, he says, his conclusion applies to digital computers, not all machines; an artefact that exactly simulates a human being would be able to think well enough. Second, he acknowledges that the Chinese room is devoid of senses for inferring meaning from the outside world, so that it is bound to be semantically bereft. Unfortunately, having launched his commentary on the problem of the Chinese room by teasing the prophets of artificial intelligence for their frequent over-optimistic statements of the potential of their machines, he will have left his listeners with the impression that no machine can think.

Many of Searle's listeners will have been reminded of other such essays, perhaps that eleven years ago in which Sir James Lighthill, on a commission from the Science Research Council, concluded that the claims then being made (chiefly at the University of Edinburgh) on behalf of artificial intelligence were either schemes for making machine computation capable of more complicated tasks or schemes for the further automation of mechanical process. There were no proposals, said Lighthill, for bridging the gap — whereupon the research council turned its stoniest face towards grant-proposals in this field, setting back an interesting area of research for the best part of a decade.

Searle's distinction between syntax and semantics is helpful as far as it goes, but that, unfortunately, is not very far. It creates but does not bridge a gap. The snide riposte to the implied jibe that the Chinese room is necessarily stupid is to ask what would happen if the man inside were smart enough to figure out what all those ideograms really meant. The truth is that there is a gap, but one that is already narrower than when the Lighthill report appeared eleven years ago. And the purpose in seeking to fill the gap is not to replace people by machines (because, cost apart, people are more adaptive to changing circumstances than machines) but to understand better how people function (and, perhaps, to improve the automation of processes as a by-product). To fail to acknowledge this is what gives philosophers a bad name with people at the bench. Let us hope that Searle does better when he comes to free will. **John Maddox**

Burkitt's lymphoma

Clues to the role of malaria

from M.A. Epstein

STEADILY accumulating evidence that Epstein-Barr (EB) virus plays a crucial role in the chain of events which causes Burkitt's lymphoma in endemic zones culminated in the compelling findings of the World Health Organization's prospective study in Uganda¹. The virus seems to play its part by infecting and thereby 'immortalizing' a pool of B lymphocytes. Their continuous cell divisions facilitate any one of three characteristic chromosomal translocations that can activate the *c-myc* oncogene². However, since EB virus is ubiquitous whereas Burkitt's lymphoma has a high incidence only in remarkably restricted areas, it has long been recognized that a co-factor must determine the geographical distribution of the endemic tumours. Already fifteen years ago Denis Burkitt suggested that hyperendemic malaria was the co-factor and collected much supporting epidemiological evidence³. But possible mechanisms for its mode of action have remained obscure. On page 449 of this issue Whittle *et al.*⁴ provide the first clues.

Like all other herpes viruses, EB virus gives rise to a life-long infection to which the healthy carrier develops specific immunological responses. These are excellent at damping down the virus but never enough to eliminate it from the, as yet unidentified, immunologically-privileged site in which it persists. Thus, a delicate balance evolves. On the host side, the balance depends both on virus-neutralizing antibodies, which restrict the spread of the agent from one target cell to another, and on a full cellular immunological response, which is capable of destroying infected cells because they display viral antigens.

During primary EB virus infection, natural killer cells and other non-specific T cells may be of immediate importance. But thereafter, specific cytotoxic T lymphocytes (which are HLA-restricted) play a central role in maintaining life-long surveillance of the viral carrier state⁵. The very great importance of this surveillance is shown by the dire consequences when it fails: genetic defects lead to death from uncontrolled polyclonal proliferation of EB virus-immortalized cells, and widespread virus replication follows primary infection. Therapeutic immunosuppression in organ graft recipients also carries a considerable risk of malignant lymphoma⁶.

Although it has been known in general terms that malaria has profound effects on immune reactivity⁷, Whittle *et al.*⁴ now clearly demonstrate that the control of EB virus-specific T lymphocytes is dramatically impaired during acute malarial infection by *Plasmodium falciparum*. Interestingly, this impairment resembles

that of immunosuppressed renal allograft patients⁸. Moreover, while looking for changes in lymphocyte numbers that might explain the phenomenon, Whittle and colleagues have recorded further intriguing observations. Not only are T cell numbers decreased and B cells numbers increased, as might be expected, but the percentage of T-helper (T4) cells is reduced in relation to T-suppressor (T8) cells, giving a low T4/T8 ratio. It is not clear how malaria brings about these changes but the loss of T4 cells is not due to cytotoxic antibodies present in the acute phase serum.

A reduced T4/T8 ratio is one of the key hallmarks of acquired immune deficiency syndrome (AIDS), in which T4 lymphocytes are infected and destroyed by the retrovirus known as LAV⁹ or HTLV III¹⁰. Whittle *et al.* make the interesting speculation that a latent retrovirus of this group, after activation by malaria, might similarly account for the depressed T4/T8 ratios in their malaria patients.

However the damage to specific T-lymphocyte control in malaria arises, it is not difficult to envisage that in the hyperendemic disease, with its repeated attacks throughout each and every year, such damage will favour the unrestrained growth of EB virus-carrying B cells in a substantial number of individuals. A firm

immunological explanation for the geographical coincidence of endemic Burkitt's lymphoma with hyperendemic malaria has thus been provided by experiments of Whittle *et al.*, even though the exact cause has not yet been identified.

Other loose ends remain to be tied up. Even where EB virus and hyperendemic malaria coexist in populations, the results of the prospective study in Uganda suggest that certain individuals are at special risk. Evidence for a genetic predisposition has not been forthcoming and should perhaps be more rigorously sought for. It has also been suggested that infection by the virus very early in life might be a predisposing factor¹¹; this too requires clarification. Alternatively, some special sequential relationship between the primary viral infection and the malarial infection could be important either in determining damage to the EB virus-specific T cells, or in providing unusual target B cells, which more readily escape from the damaged control mechanisms. □

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M.A. Epstein is at the University of Bristol Medical School, University Walk, Bristol BS8 1TD, UK.

Mathematics

Five bodies to infinity

from Ian Stewart

TYCHO Brahe's observations of planetary motion led Johannes Kepler to the idea that planets orbit the Sun in ellipses. Isaac Newton showed that this would follow from an inverse square law of attraction. A fully rigorous and complete treatment of the problem of the motion of two bodies under Newtonian gravitation was given by Leonhard Euler in 1744. The extension to more than two bodies has proved considerably less tractable. While excellent approximate methods exist (and are widely used in astronomy and space exploration), general theoretical insights seem hard to come by. The problem is deep and difficult, the phenomena are complex. Just *how* complex is shown by J. Gerver in the *Journal of Differential Equations* **52**, 76 (1984).

Gerver gives a heuristic but convincing argument for the existence of configurations of five point masses which, under their mutual Newtonian gravitational attraction, fling themselves out to infinity in a finite time. This relates to an important theoretical issue: the possible occurrence of

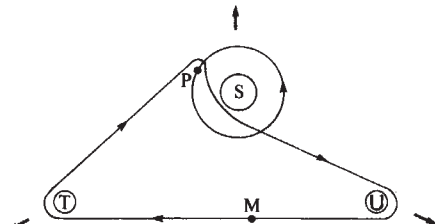
singularities, that is events beyond which the motion cannot be continued within the mathematical model. In 1897, Paul Painlevé proved that for three bodies, every singularity must be a collision. Collision singularities are fairly well behaved: in fact if only two bodies collide at a given instant, then the motion can be 'desingularized' by assuming elastic collisions. But Painlevé also suggested that with more than three bodies there might exist much nastier types of singularity such as bodies escaping to infinity in a finite time, or oscillating ever more violently.

In 1970, H.J. Sperling proved that in order to have a singularity without collisions, at least one body must escape to infinity; and seven years later D. G. Saari showed that one of the escapees would have to oscillate wildly. He also showed that, in the case of four bodies, they would have to approach a straight line arrangement as time increased. In 1974, J. Mather and R. McGehee, studying an idealization, showed that escape to infinity in finite time

could occur for four point particles constrained to lie on a line and colliding elastically. The process involves an infinite number of energy transfers, happening ever more rapidly: conservation of energy does not rule out such escapes.

Gerver argues that in the (planar or three-dimensional) five-body problem, non-collision singularities should exist. The proposed mechanism is ingenious. Imagine three massive 'stars' S, T and U represented by point masses. Make S slightly heavier than T and U. Arrange all three stars in a triangular constellation with an obtuse angle at S, and start them out in such a way that the triangle expands at a uniform rate while maintaining its shape. Add a tiny 'meteorite' M, which orbits round the outside of all three, approaching them very closely (see figure).

As M passes each star it undergoes the same 'slingshot' effect that was used for the Voyager spacecraft journeys through the



Solar System. As a result, M gains a small amount of kinetic energy from star S, which it can then share with T and U to increase their speeds (and its own). If only there were a way also to increase the energy of S, it would be possible to make the triangle expand at an ever increasing rate, disappearing to infinity in a finite time.

Of course, conservation of energy forbids this — but there is a way out. Place a fifth 'planet', P, in a roughly circular orbit round S. Now make M gain its energy at the expense of P and transfer some of it to S via a 'zig-zag' slingshot path. Otherwise proceed as before. Now, as time increases, the triangular constellation expands ever faster but does not change its shape, M moves ever more rapidly in its triangular orbit, and the orbit of P descends ever closer to S. As a result, all five bodies escape to infinity in a finite time.

This scenario requires a very delicate balancing act. For example, planet P must be in a suitable position for the zig-zag slingshot not just at the first pass, but at every pass; its orbit must stay roughly circular; and the triangle of stars must stay stable. Furthermore, the energy transfers must be handled in such a way that the increase in the speed of the meteorite as it traverses the constellation is greater than the rate at which the constellation grows, so that the orbital period of the meteorite shrinks. Gerver argues plausibly (but heuristically) for the existence of a set of initial conditions that keeps all of these problems under control. He adds that a calculation 'nearly one hundred pages long' give a rigorous proof that the 'Great Escape' does occur in an analogous model

problem, in which some particles are massless and the meteorite undergoes elastic collisions instead of slingshots. The full result might be proved in "hundreds of pages of calculations". A better approach might be to seek new methods to make the arguments more rigorous.

In the real Universe, of course, additional bodies would interfere with this deli-

cate scenario, and relativistic effects would change the results. But Gerver's idea is a bold and imaginative thrust at the heart of a celebrated problem, and a challenge to the current mathematical techniques. Isaac Newton would have loved it. □

Ian Stewart is in the Mathematics Institute, University of Warwick, Coventry, CV4 7AL, Warwickshire, UK.

Radiocarbon dating

New World colonized in Holocene

from Richard Burleigh

A PAPER by J.L. Bada *et al.* on page 442 of this issue describes the application of the new accelerator mass spectrometric (AMS) technique of radiocarbon dating to human skeletal remains from California. The dating of the remains to the Holocene is important for two reasons: first, it adds to the increasing body of evidence placing the human colonization of the New World in this period; and second, with much wider relevance, it provides a striking demonstration of the power of AMS.

The time of first arrival of humans in the New World has long been a matter for controversy. During the earlier decades of this century any claims for the antiquity of apparently early human skeletal remains discovered in the Americas were hotly contested, notably by the physical anthropologist Ales Hrdlička. At their most vehement, Hrdlička's views would not allow the presence of humans there for more than a few millennia, although eventually he was prepared to concede a possible time of arrival of 10,000–15,000 yr ago. The most widely accepted view today is that human groups first entered North America some 12,000–13,000 yr ago after the ice had begun to retreat at the end of the last glaciation. They most probably came across a land bridge from Asia (Beringia) rather than by a coastal (sea) route. On this model, if human remains very much older than about 12,000 yr before the present (BP) were to be found in the Americas, it would suggest that entry had occurred during the previous interglacial, some 70,000–100,000 yr BP; there is no fossil or lithic (artefact) evidence in support of this. In other words, the first people probably arrived in the Holocene (postglacial) rather than during the Pleistocene. Conventional radiocarbon dating over the past 25 years has contributed support for this time scale for the appearance of humans in North America and for their subsequent very rapid spread southwards.

Racemization dates of up to 50,000 or even 70,000 yr BP, obtained by Bada and his colleagues at the Scripps Institution in the mid-1970s for amino acids extracted from human skeletal remains from California (notably those from Del Mar and Sunnyvale), were therefore in immediate conflict with accepted views of

the antiquity of the human population of North America deduced from radiocarbon evidence. On the other hand, although these dates seemed incredible they could not be lightly dismissed. The measurements were carefully made, apparently independently 'calibrated' by a radiocarbon date of 17,000 yr (UCLA-1233A) for a human skull from Laguna Beach, California (itself an unexpectedly early date), and, above all, were direct analyses of the skeletal remains themselves.

The opportunity to test the validity of these results comes with the successful development over the past seven years of the AMS technique of radiocarbon dating (Hedges, R.E.M. & Gowlett, J.A.J. *Nature* **308**, 403; 1984) which requires only a few milligrams of sample, in contrast to conventional radiocarbon techniques, where gram quantities are needed. In their current work, Bada *et al.* have used the AMS technique to date aliquots of the original amino acid extracts used to obtain the racemization dates. Their new results (which are well-supported by some conventional ^{14}C measurements of related acid-insoluble extracts) clearly show that all the remains, including the Laguna skull, are of Holocene age. This agrees with the date reported by Stafford *et al.* (*Nature* **308**, 446; 1984), of less than 4,000 yr BP obtained using AMS for a human burial from Yuha, California, that had previously been assigned a racemization age of some 24,000 yr. Using these new results, the cranium of Los Angeles Man, previously dated by the conventional radiocarbon technique to greater than 23,600 yr BP (UCLA-1430; Berger, R. *World Archeol.* **7**, 174; 1975), is most probably also of Holocene age. These new results remove a puzzling anomaly that was in conflict not only with a model built very carefully over a long period from many independent (archaeological, geological, environmental and climatic) lines of evidence, but also with the observation that these human skeletal remains are essentially modern in appearance and show few if any of the traits that might be expected in the representatives of earlier populations. □

Richard Burleigh is in the Research Laboratory, The British Museum, London, WC1B 3DG.

Geology

Death of an ancient ocean

from Peter Gambles

THE Caledonide Orogen is the name given to the mountain belt, now torn apart by the modern Atlantic ocean, that was formed by the collision of the continental plates of North America, Africa and Baltica between about 550 and 350 million years ago. Stretching from Spitzbergen and Greenland in the north, to the Mauritanides and the Gulf of Mexico, it is one of the longest such belts in the world. The broad outline of the plate tectonic movements that formed the mountains has been known for some time, but their reconstruction still resembles a child's drawing with straight-edged continents closing over a smooth ocean floor. A glance at any globe will show that this is not the real world. Oceans are dotted with mid-ocean ridges, volcanic arcs and islands of both oceanic and continental crust; moreover, continental margins are far from straight. The exciting challenge now was made clear at the symposium on 'Evolution of the Caledonide-Appalachian Orogen', University of Glasgow,

3-8 September 1984. It is to integrate the wealth of data from traditionally distinct disciplines within earth sciences into a more realistic model of this major episode in the Earth's history.

The fashionable concept of suspect or allochthonous terranes is part of the new approach to plate tectonics. In essence, such a terrane is a sizeable chunk of continental crust, the geology of which is incompatible with that of adjacent areas. The Western Cordillera in the United States and Canada is now thought to comprise a multitude of such microcontinents. Many of these have moved hundreds of kilometres, largely parallel to the continental margin. By contrast, some of the terranes in the Appalachians have been transported from one continent to another across the oceans.

To establish the links between the Appalachian terranes and those now on the opposite side of the Atlantic, geologists must rely on the traditional methods of

comparative stratigraphy, using as a standard the Newfoundland portion of the orogen — the Humber, Dunnage, Gander and Avalon zones. Of these, the Avalon is probably the most significant in terms of the evolution of the mountain belt, and has proved useful, for example, in examining the very earliest development of the Caledonide Orogen. During the latest Precambrian (700–580 Myr), the eastern margin of North America underwent a large degree of crustal extension (D.W. Rankin, USGS, Reston). This is indicated in the geological record by the presence of basaltic dykes, forming up to 50 per cent of the exposed rocks, and by the extrusion of continental tholeiitic and alkali basalts. However, contemporaneous volcanism in Avalon was calc-alkaline, and so not related to rifting, suggesting that Avalon was not part of North America. Moreover, the distribution of fauna, especially trilobites (L.R.M. Cocks, British Museum and W.S. McKerrow, University of Oxford), favours closer links of Avalon with Gondwana (a supercontinent comprising today's southern continents including Africa) than with America during the early Cambrian (570 Myr). To the north of the continental fragment lay a number of ridges and basins such as the Irish Sea

THE evolution of the mountainous Caledonides of Scandinavia and Britain, the Appalachians of North America and Canada and the Mauritanides of North Africa has been the subject of a 10-year programme of geological research entitled 'The Caledonide Orogen'. Organized jointly by Unesco and the International Union of Geological Sciences, it has been led by a group of Norwegian geoscientists. Together over 200 specialists from countries bordering the present north Atlantic have carried out nationally financed research projects and exchanged results at a series of symposia held in conjunction with field studies.

The final symposium of the project was one with a difference in that the entire orogen was studied in terms of four time slices rather than in the more conventional divisions into subject specializations. Some of the coordinators of these sessions had clearly done their homework, while others, for one reason or another, were forced to rely on lecturers who had only a vague knowledge of what the Caledonide Orogen was and even less knowledge of its present day geography.

The scene was set by the geophysicists, whose models of the earth, based partly on data beyond the reach of the field geologist, must be tested against the field evidence. The two approaches sometimes conflict. For example, fossils and sediments may indicate a warm climate, while the geomagnetic palaeopole position suggests the contrary. In my view the evidence for an equatorially placed American plate during the Ordovician

period and for a high latitude position for Baltoscandia (~60°S) during at least the first half of the period, is overwhelming. The majority of geologists are prepared to accept this. What is not certain, however, is the relative positions of both plates and that of the African. Did they meet along a so-called triple junction, and were Africa and Baltoscandia separated by the so-called Tornquist Sea?

Meanwhile the palaeontologists continue to provide dates (with an accuracy of one million years in some instances) and information which both startles and irritates. Yet they cannot be disregarded, even when they happen not to fit some of the many, and often conflicting, dates produced at vast expense by radiometric methods. I was amazed at how uncritically some of the radiometric dates were used to fit the geological models. A fossil cannot be reset nor can it be destroyed (unless lost). It can be wrongly interpreted but it is a firm piece of tangible evidence. The Caledonide Orogen project has done more than any other to encourage palaeontologists to provide answers to questions posed by field geologists rather than other palaeontologists. Their diligent collecting has provided a wealth of material from the most unlikely rocks; shelly fossils have even been found in volcanic ashes derived from oceanic islands along the length of the belt.

Perhaps the most successful part of the whole project, and certainly the most exciting session of the symposium, concerned geological maps. From the inception of the project, specialist study groups were set up with the specific goal of

producing a series of 1:1 million scale geological maps of the Caledonide-Appalachian Orogen. In addition to the more usual maps of geological structures, the distribution of volcanic and plutonic rocks, and the relationships between sedimentary rock types and faunas, there were a number of more adventurous projects. These covered the timing of deformation and age of metamorphism in various parts of the orogen, and the relationship between basement rocks and their cover, topics almost designed to be controversial. From the start, a standard time scale was agreed on, as were symbols and colours. The end result is that British and Irish geologists cooperated to provide three handsome new maps, on sale at the symposium, and Norwegian and Swedish geologists jointly produced a map with boundaries that actually coincide where they cross the state border. Regrettably, a number of the thematic maps are still provisional and there is doubt as to whether funds to publish them will be forthcoming.

When the final speech of the symposium declared the end of the project, those of us who thought it an enjoyable and worthwhile experience have the prospect of another to look forward to. 'Suspect terranes', as it may be called, will be considered by the board of the International Union of Geological Societies in 1985. □

David L. Bruton, Palaeontological Museum, University of Oslo, Sars Gate 1, Oslo 5, Norway.

landmass and the Leinster-Manx and Welsh Basins, separated from America by a major ocean — the Iapetus. The history of the Caledonide Orogen in this region thus appears to be concerned with the closure of the Iapetus Ocean, but not, as previously thought, with its opening.

Between Avalon and Africa (Gondwana) was a narrow basin termed the Cadomian Ocean. Palaeomagnetic data suggests that during the late Cambrian–early Ordovician (520–480 Myr), Gondwana rotated about a pole in eastern Africa, thereby closing up this seaway (C.R. Scotese, University of Texas, Austin). This is in agreement with the sedimentological evidence for infilling of the basin by shallow water sandstones (F.L. Schwab, Washington and Lee University).

From the middle Ordovician (470 Myr) onwards, a number of fragments of island arcs, formed by subduction of the Iapetus Ocean under North America, were split off from the continent by inter-arc basins, which in turn were rapidly filled with sediments derived from volcanoes (C.J. Stillman, Trinity College, Dublin). All this material was variously subducted or obducted as it moved northwards towards the craton. Pieces of oceanic crust, such as the Bay of Islands ophiolite, were trapped during this pulse of tectonic movement but interestingly very little geochemically-normal open oceanic crust from Iapetus has been found. The rocks of the continental margin and Humber zone were deformed and metamorphosed during the middle Ordovician, but the absence of such effects from the Dunnage and Gander zones suggests that they were by then accreted to Avalon as it moved northwards, impacting with North America by the late Devonian (370 Myr) and opening the Theic Ocean behind it.

This style of plate tectonics — the shuffling of continental blocks or suspect terranes — can be traced from the southernmost parts of the Appalachians to northern Scotland. Across the North Sea, the picture is dramatically different. The Caledonides in Scandinavia are a classic example of large-scale thrust tectonics. The mountain chain is built up of four allochthonous units that are believed to have travelled considerable distances — a theory familiar to Alpine geologists but less readily accepted by the British

Cocks presented palaeontological evidence indicating that at least the upper two nappes in Scandinavia were originally part of the North American plate. Ophiolites are restricted to these two sheets which, in the manner typical of nappe stacks, originated furthest from their present position. All the nappes contain volcanic rocks, geochemical study of which (H. Furnes, University of Bergen) shows that the lowest three were involved in rifting and only the uppermost contains calc-alkaline sequences characteristic of an island arc. The Baltic coast of the Iapetus Ocean thus appears to have been a passive plate tec-

tonic margin, with the oceanic crust being subducted under North America or Greenland. The Scandinavian Caledonides are considerably deformed and metamorphosed, the emplacement of the nappes being one of the last tectonic events. R.D. Dallemeyer (University of Georgia) has used ^{39}Ar – ^{40}Ar dating of individual minerals (in itself a technical achievement) to demonstrate very neatly that in Scandinavia there were two distinct orogenic phases, termed the Finnmarkian (500–460 Myr) and the Scandian (420–400 Myr).

During the early Silurian, sheets of Iapetus sediments and crust were thus being thrust eastwards onto the Baltic craton. What of the opposite side of the ocean? In east Greenland, K–Ar ages (also an indicator of deformation and low grade metamorphism) of 445–410 Myr are recorded in the main fold belt (R.J. Tracey,



Distribution of the Caledonide-Appalachian-Mauritanide mountain chain (in black) before the opening of the present Atlantic.

Yale University) and it is now thought that much of the material in Greenland which underwent Caledonian metamorphism may in fact be allochthonous on Precambrian (Grenville) basement.

As McKerrow pointed out, the problem that then arises is how, during the Scandian orogeny, could nappes have been thrust eastwards on to the eastern margin of the Iapetus Ocean (Norway) at the same time as others were being forced westwards on to the western margin (Greenland)? His elegant solution is to split the subduction zone down which the crust of the Iapetus was disappearing into two, by means of an east–west oriented transform fault, across which the direction of subduction changes. A large amount of strike-slip motion is further involved, with the various parts of Spitzbergen being strung out on either side of the subduction zone dipping westwards under south Greenland. W.B. Harland (Cambridge University) argued that east Spitzbergen was positioned off east Greenland in the early Palaeozoic, while the geology of west Spitzbergen resembles that of north Greenland more closely. The fascinating possibility arises that Norway and Greenland swapped thrust sheets and that Spitzbergen was squeezed out northwards as the Iapetus closed.

So what separated the northern part of

the Caledonide Orogen from the southern part? The answer lies in the Tornquist line — one of the major ancient lineaments to have cut the crust of the continents since the Precambrian and which stretches from the Black Sea through Poland and Denmark to the North Sea.

Most Western geologists believe that there was a Tornquist Sea (or Rheic Ocean) which separated Baltica from Gondwanaland (including south Europe) until the latest Ordovician (440 Myr). Their belief is primarily based on the presence of two distinct faunal provinces which broke down only in the early Silurian. Unrefined palaeomagnetic data seem to suggest that the Tornquist Sea existed well into the Devonian (380 Myr). Polish geologists, however, find no evidence of deformation due to the closure of this putative seaway (B. Lewinski, Polish Geological Survey), though they have identified a major fault at depth from seismic studies, and recognize various phases of movement along the Tornquist line. Lewinski suggests that the faunal provinces can be accounted for by other barriers, such as climatic belts.

As can be seen from the Mediterranean, the closing stages of the collision of major plates are very complex. Some areas consolidate whilst small basins develop in others, perhaps with minor amounts of oceanic crust flooring them. J. Rodgers (Yale University) suggested that the ocean between Avalon and Africa still contained islands of 'Avalonia' after the main fragment was consolidated to North America. This is likely also to have been the case in the Theic Ocean and perhaps pieces of the European continental mosaic can be taken and placed in the Tornquist gap, thus eliminating the need for a true ocean. (The Tornquist line runs straight into the North Sea, which contains no normal oceanic crust. If the North Sea were to close, what evidence would remain of its existence in 400 million years time?)

The Caledonide Orogen was thus not simply the closure of the Iapetus Ocean. Rather, a picture emerges of a southern realm dotted with large fragments of continental blocks forming its margins, which were jostled about as Gondwana pushed relentlessly towards America. At the same time the northern arm of the Iapetus closed progressively from the north, thrusting great thicknesses of rocks onto the cratons on either side. The final locking of the three continents of Gondwana, North America and Baltica in the late Devonian (370 Myr) produced the supercontinent of Pangaea. But within a few tens of millions of years, far less than the lifespan of the orogen, Pangaea started to break up with renewed rifting in the Oslo and Rhine regions, leading to the opening of the Atlantic Ocean. In some ways, therefore, modern geology is but a continuation of the Caledonide Orogen, as the dance of the continents goes on. □

Peter Gambles is an assistant editor of *Nature*.

Drosophila development

Smell taste and rhythm

from Michael Ashburner

ALREADY hurt by the news that, with respect to the development of its nervous system, *Drosophila* is but a small grasshopper¹, drosophologists assembled in Crete last summer* learnt that the central nervous system of their favorite fly may not be so different from their own.

M. Heisenberg (University of Würzburg), reviewed the evidence from his laboratory² that the mushroom body, a region of the brain of insects thought to be important for conditioned behaviour, is developmentally plastic. The number of mushroom body fibres normally increases in the first week of adult life. Extraordinarily, this increase does not occur if the flies are kept in a deprived environment, for example alone rather than with their fellows. Heisenberg's group have isolated mutations that effect the mushroom body. In one, the Kenyon cells — neurones intrinsic to the mushroom bodies — degenerate in female larvae. Although flies with mushroom body defects can smell, they have impaired olfactory conditioning. What is the significance of this? A clue may come from flies that are homozygous for mutations of the *tra-2* gene. These flies develop as phenotypic males even if they have two X chromosomes.

A temperature sensitive allele of *tra-2*, *tra-2^{ts}*, allows female development at 16°C but male development at 29°C. Strangely, however, if 16°C females are kept for six days at 29°C as adult flies, they begin to show male sexual behaviour³. It seems to be a reasonable hypothesis that the behavioural plasticity revealed by the experiment with *tra-2^{ts}* and the morphological plasticity seen in the mushroom bodies are related, suggesting a role of olfactory conditioning in the sexual behaviour of the flies.

Drosophila is a sugar-lover, with separate receptors for pyranose, furanose and trehalose on its feet and labellum (O. Siddiqui, Tata Institute, Bombay). Mutations that affect chemoreception may either influence the receptors themselves, such as *gust A* which eliminates the pyranose response, or act centrally. *Gust R* is an interesting member of the latter class, since it creates a fly that is quite indifferent to all sugars yet is abnormally attracted by salt. Siddiqui finds that the response of the sugar and salt receptors is normal and explains the phenotype as a result of central processing of inputs from the salt receptors as if they had originated from sugar receptors.

Many years ago R. Konopka, then at California Institute of Technology, ran a heroic hunt for X-linked mutations that

affect the circadian rhythm of eclosion of flies from the pupal case. Wild type flies eclose with a rhythm of 24 hours. Konopka found three mutations — one abolished all evidence of rhythm, one lengthened the period of the rhythm to 29 hours and one shortened it to 19 hours. All three were alleles of a single locus — called *per*, for *period*⁴. Both M. Young (Rockefeller University) and M. Robash with J. Hall (Brandeis University) have now cloned *per*. Dramatically, the Brandeis group find that a 0.9 kilobase transcript from the *per* locus is at least 10-fold more abundant at noon than at midnight, even in flies that are kept under constant dark conditions. Moreover the arrhythmic phenotype of the *per*⁰ mutation has been corrected with cloned *per* by germ-line transformation. Of course, we are still some way from understanding how the *per* gene product controls the circadian behaviour of flies — let alone its role in other rhythms⁵.

R. Wyman and J. Thomas (University of Yale) described the isolation of *Drosophila* mutants that cannot escape the fly swatter's swat. Two of their mutations disrupt the synapses between the giant fibre, which descends from the brain, and the jump motor neurone: *bendless* affects the presynaptic cell — the giant fibre's 'bending' branch, which should make the synapse, is simply missing; *passover* affects the postsynaptic cell — the central branch of the jump motor neurone passes right over the giant fibre without making a proper synapse⁶. Wyman speculates that the wild-type alleles of these genes encode molecules required for cellular recognition during neurogenesis. The wonder, of course, is that the mutant phenotype is so specific.

Growth and development of *Drosophila*, in common with all other insects, is coordinated by a steroid hormone, ecdysterone. Four genes that respond to ecdysterone have now been cloned. Two correspond to the well-known puffs of the larval salivary gland 74EF and 75B (D. Hogness, Stanford University); the other two code for proteins synthesized as an early response to ecdysterone by Kc tissue-culture cells (P. Cherbas, Harvard University). The former pair, called E74 and E75, are both gigantic. E74 has a 62 kb primary transcript, processed by the removal of at least six introns to a putative mRNA of 5.9 kb with a mere 0.9 kb open reading frame, and E75 has a primary transcript of at least 50 kb which is processed to 5 and 6 kb products. Following induction by ecdysterone, these RNAs accumulate and then, within 2–4 hours, decline in a way that closely parallels the behaviour of the salivary gland puffs.

E74, at least, does not appear to be tissue specific. One of the genes described by Cherbas can produce two proteins by means of alternative splicing of the gene transcript and has at least four other transcripts which show a high degree of tissue specificity. It is conceivable that there is a family of related ecdysteroid-induced proteins, differences in which may play a role in the specificity of response seen at the cellular level. Intriguingly Cherbas has evidence for symmetrical transcription from both strands of ecdysterone-inducible genes.

No Kolybari meeting can be considered complete without discussion of heat shock and transposable elements. One chromosomal puff (93D) produced by heat-shocking *D. melanogaster* has long been an enigma, for all the evidence has suggested that it does not code for proteins. Moreover it alone is induced by benzamide⁷ and is also full of large ribonucleoprotein granules. At last 93D has been cloned by both B. Hovermann and E. Bautz (University of Heidelberg) and by M.L. Pardue (MIT). The region transcribed after heat shock is made up of tandemly repeated 280 bp units cut by the enzyme *TaqI* and little else. The homologous puff of *D. hydei* (48C) has a 115 bp repeat that shows no homology with that of 93D, despite the fact that the regions which flank the repeats are homologous in these species. On heat shock, the 93D puff accumulates a ribonucleoprotein-associated antigen known as P11. Bautz suggests that perhaps 93D is a refuge for mRNAs, protected by P11, during the time that their transcription is blocked by heat shock.

Applications of P element-mediated germ-line transformation of *Drosophila*⁸ include the rescue of a mutant phenotype with a cloned DNA fragment, thereby confirming the isolation of the mutation's wild-type allele. A dramatic extension of this method was discussed by V. Pirrota (EMBL, Heidelberg), who has rescued a mutation by transformation with a 40 kb fragment on a cosmid vector with P element ends. One of the surprises of the work with transformed *Drosophila* strains has been that most genes function more or less normally in development regardless of the position of their chromosomal integration. Some influence of position on expression can however be seen. For example, G. Richards (CNRS, Strasbourg) has an integrant of the salivary gland glue gene *Sgs-3* that is almost inactive despite (or because?) of its location within 0.8 kb of the resident *Sgs-3* gene.

Richards and others are using transformation to identify *cis*-acting sequences that are required for proper gene expression. Thus, the alcohol dehydrogenase gene is expressed in larval salivary glands after transformation with a construct of the gene and the up-stream noncoding sequence of a salivary gland glue gene (S. Beckendorf, University of California, Berkeley). However, there is also gene

* The fourth EMBO workshop on 'Molecular Biology and Development of *Drosophila*', Orthodox Academy of Kolybari, Crete, 24–29 June 1984. The sessions on homeotic genes were reported in *Nature*, 20 September, p.214.

expression in the gut, so this is not simply a case of the effects of a tissue-specific enhancer.

The analysis of *cis*-acting elements required for the tissue and temporal specificity of gene expression is in its early days. Yet it would be safe to predict that it will be a topic that will dominate discussions at the next Kolymbari meeting, that will take place in 1986. □

Cell biology

Actin-binding protein evolution

from Thomas D. Pollard

READERS of *Nature* must find the seemingly endless proliferation of actin-binding proteins a bit overwhelming. They might well ask when will it end and how does the paper on page 424 by Maruta *et al.*¹, describing the possible evolutionary origin of *Physarum* capping proteins, fit into the story?

Let us consider first where the field stands and where it might be headed. With some rare exceptions, actin is present in the cytoplasm at concentrations up to several hundred micromolar, higher than that of any other protein. Such concentrations of purified actin would polymerize very rapidly, leaving less than one per cent in monomeric form at physiological salt concentrations. In cells, only part of the actin is polymerized and a repertoire of regulatory proteins appear to govern the time, place and extent of polymerization and to organize the filaments into higher-order structures (see Table). Specific regulatory proteins probably exist for each step in the assembly process, including nucleation, growth at the two ends of the polymer and fragmentation of polymers.

Although more than 60 such actin-binding proteins have been described from various tissues, each passing month makes it clearer that they will eventually all fit into a limited number of families. (The problem, as one wag has put it, is that scientists would rather use each others' toothbrushes than their nomenclature, hence there is usually more than one name for similar proteins.) More importantly, I now feel it is safe to predict that representatives of at least most, and perhaps all, of these families are present in all non-muscle cells. Consequently, information about a new actin-binding protein isolated from *Acanthamoeba*, *Dictyostelium* or *Physarum* (the three favourite 'primitive' cells for such studies) is likely to be informative about homologous proteins in vertebrate cells.

With this in mind, what have we learned from the new work of Maruta *et al.* on *Physarum* capping proteins? The proteins in question are part of a family of Ca²⁺-sensitive proteins that can both fragment actin filaments and block their 'barbed' (fast-growing) end. (There are three other

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Michael Ashburner is in the Department of Genetics, Cambridge University, Downing St., Cambridge CB2 3EH, UK.

families of these proteins that bind to the ends of actin filaments; see the Table.) Intriguingly, Maruta *et al.* have found that three actin filament-capping proteins — fragmin², Cap 42(a) and Cap 42(b)³ — share some features with actin itself: all four proteins bind ATP; there is partial cross-reactivity among antibodies to the four proteins; and pairs of the proteins have similar peptide maps. There are

capping protein has retained the sites used by actin to bind to the end of the filament but has lost (or modified) the sites used by actin to bind the next subunit in the polymer. Such a modified actin would be ideal for capping the end of the polymer.

Have other actin-binding proteins evolved by modification of an actin gene? Although not conclusive, most evidence suggests that they are not related to actin. For example, the sequence information available on profilin⁴ and gelsolin⁵ reveal no similarity to actin. Fingerprints of profilin, actophorin, capping protein and actin from *Acanthamoeba*⁶ are clearly different. Finally, I know of no other case where an antibody to actin-binding proteins cross-reacts with actin; in fact, with the exception of some antibodies to spectrin⁷, most antibodies to actin-binding proteins show no reactivity with proteins outside their own class.

The organization of the actin system will be understood only when the catalogue of components is completed and the mechanism of action of each protein has been elucidated in detail. Current work suggests that these mechanisms will be

Distribution of actin-binding proteins					
Class	Families	Subunit MW	Protozoa	Other lower eukaryotes	Vertebrates
Bind actin monomers	Profilin	12–15K	+	+	+
	Depactin/actophorin	16–20K	+	+	+
Bind end of actin filaments	Capping protein	29K + 31K	+	+	+
	Fragmin/severin	40–45K	0	+	+
	Accumbent	65K	0	0	+
	Gelsolin/villin	90–95K	0	0	+
Bind along actin filaments	Tropomyosin	30–40K	0	0	+
Cross-link actin filaments to each other	Gelactins	23–38K	+	0	0
	Fascin/fimbrin	55–70K	0	+	+
	α -Actinin	90–100K	+	+	+
	Actin-binding protein/filamin	250K	0	0	+
Cross-link actin filaments to other structures	Brush border 110K	110K	0	0	+
	Spectrin	220–260K	+	+	+
	Microtubule-associated protein 2	~260K	0	0	+
Myosins	Myosin/myosin-II	175–220K	+	+	+
	Myosin-I	125–130K	+	0	0

Actin-binding proteins are grouped into classes by their established properties and subdivided into families according to physical properties and presumed mechanisms of action. This classification is arbitrary and will probably be modified as more data become available. +, the protein has been identified in these cells; 0, the protein has not yet been identified in these cells (or rarely, for example in the case of tropomyosin in protozoa, the protein has been sought but not found). MW, molecular weight.

equally clear differences: actin and Cap 42(b) bind to DNase I but fragmin and Cap 42(a) do not; there are kinases specific for subsets of these proteins; and nucleotide-binding properties of the proteins differ. The main difference, of course, is that actin polymerizes whereas the capping proteins interfere with this process by blocking the fast-growing end.

The fascinating suggestion of this work is that at least one class of actin-binding proteins has evolved by duplication and modification of the actin gene itself. This idea will have to be substantiated by examining the primary structure of the proteins and their genes but, if true, argues for a clever economy in the evolutionary processes. Perhaps the actin gene has been modified selectively, so that the resulting

complex. But this will only be the beginning, because the actin-binding proteins form a highly interactive regulatory system. Consequently, direct tests at the cellular level will be necessary to assign physiological function. □

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Thomas D. Pollard is in the Department of Cell Biology and Anatomy, Johns Hopkins Medical School, Baltimore, Maryland 21205, USA.

Particle physics

Computer simulations of quark and gluon interactions

from D. J. Wallace

COMPUTATIONAL methods have a particularly important role to play in the many areas of science and engineering where current research and design problems are too complex to permit analytical study. The rapid expansion of international interest in numerical simulation is due in large measure to the increasing speed and memory of computers. Conversely, many problems demand far greater power than even present "supercomputers" can provide, a realization that is driving the development of yet more powerful machines. One of the most active areas is the study of the strong nuclear force, involving the numerical simulation of the interactions between quarks and gluons on a four-dimensional lattice, approximating continuous space and time.

Typically, the numerical approach adopts a discrete representation of the system under study. For example, in the case of fluid flow in systems of pipes, the velocity field $\mathbf{v}(\mathbf{r}, t)$ which depends on the spatial position $\mathbf{r}$ and time t , is approximated by the velocities $\mathbf{v}_i(t) = \mathbf{v}(\mathbf{r}_i, t)$, $i = 1, 2 \dots N$ at points $\mathbf{r}_i$ in the fluid. The differential equations obeyed by the velocity field $\mathbf{v}(\mathbf{r}, t)$ are approximated by difference equations obeyed by the $\mathbf{v}_i$. Since we now have a finite set of variables, these difference equations can be solved on a computer. Clearly, if the set of N points chosen is sufficiently dense, and if the starting equations incorporate all of the relevant physics (in this case, elastic properties, viscosity, compressibility, thermodynamic properties . . . the equations can become complicated!) then, in principle, arbitrary numerical accuracy can be obtained.

As in all cases, a key ingredient for the successful simulation of the strong nuclear force is knowledge of the fundamental equations describing the force. It is now widely accepted that these equations must be formulated not in terms of some force that binds protons and neutrons within the nucleus, but at a deeper level, in terms of the force between more fundamental constituents, out of which protons and neutrons are built. Just as the electric force binds nuclei and electrons together to form neutral atoms which can then combine to form molecules under the residual Van der Waals forces, so we now understand that the strong nuclear force binds more fundamental constituents together to form protons and neutrons; nuclei are then "molecules" formed by the residual strong force between protons and neutrons. This theory is called quantum chromodynamics (QCD) and describes how the so-called

quark constituents interact with one another through the exchange of "gluons".

Apart from experimental evidence from high energy scattering and from model calculations, QCD is aesthetically attractive because it belongs to the same class of theories as the electromagnetic and weak nuclear forces, namely it is a gauge theory. However, the usual weak-coupling perturbation theory, which has been so successfully applied to the electroweak interactions, breaks down for QCD because the effects of interactions are not small. Indeed, perturbation theory has to fail because free quarks, on which the theory would be based, are not observed experimentally; somehow they are permanently confined within observed particles such as the proton. The solution of this confinement problem in QCD should enable one to calculate the masses of the numerous "hadronic" particles such as the proton, just as properties of atoms and molecules can, in principle, be calculated in terms of the electric and magnetic forces among their nuclei and electrons.

The radical step of abandoning the continuity of space and time and formulating QCD on a discrete lattice of points was first made by K.G. Wilson of Cornell University ten years ago. In the currently most popular version of this approach, the quarks are allowed to sit at sites of a regular, usually hypercubic, four-dimensional lattice (approximating space-time) with the gluon variables occupying the links joining the adjacent sites. The theory then requires that the quark and gluon variables are allowed to interact and come to an equilibrium. One can then calculate the propagation, that is the motion on the four-dimensional lattice, of the combination of quarks that make up a particular particle. The form of the propagation depends on the mass of the particle which can hence be estimated from the numerical simulation. The whole problem is somewhat akin to, but much more complicated than, the simulation of the Brownian motion of a particle of dust in a fluid under the chaotic bombardment of the molecules in the fluid.

This is an enormously demanding computational project for many reasons, several of which are endemic in simulation problems. First, the quark and gluon variables are rather complicated; at least 50 variables must be stored at each lattice site in any realistic simulation. Second, we are interested in the limit in which the lattice spacing is very fine compared with the particle of interest. Since the computer must simulate a box of space and time large

enough to contain the proton without its feeling the edges of the box, we need to have many lattice sites. Third, just as small changes in the shape of a cloud may occur rapidly but the overall aspect tends to occur much more slowly, so it takes many steps on the computer to simulate the behaviour of a very correlated object, such as the proton, which extends over many lattice sites. Fourth, the problem is four-dimensional so that increasing the fineness of the lattice by a factor of 2 requires 2^4 more sites; in fact the problem is much worse since the proton now extends over more lattice sites, so that the correlation problem demands yet more computer time. Finally, since the quarks are fermions and must obey Fermi statistics, the quark variables cannot be directly represented by numbers and new algorithms must be developed to deal with them; to date, these are still very costly in terms of computer time.

Notwithstanding these difficulties, very considerable progress has been made in extracting strong interaction physics from simulations based on lattices, currently with a maximum of typically $16 \times 16 \times 16 \times 16$ sites. There are now good quality numerical data showing that the effective force between a quark and an anti-quark changes from the Coulomb form at separations smaller than the proton size to a constant (attractive) force at large separations; confinement then emerges because infinite energy would be required to isolate a single quark. The theoretically expected "scaling" behaviour as the lattice is made finer and finer is beginning to be exposed, to a numerical accuracy of some ten per cent. The reduction of statistical and systematic uncertainty in particle mass predictions is likely to be reduced to this level in the round of calculations now being undertaken. Practical suggestions for the calculation of particle decay and scattering have also been made.

There is a further dimension to the computational aspects that is central to the feasibility of these and many other simulations. To appreciate the point one must realize that the increase in the speed of computers by a factor of roughly a million in the past thirty years has been achieved not only because of the increasing miniaturization and speed of the electronic components but also because machines are designed increasingly to do many things simultaneously, that is in parallel. The rates of tens to hundreds of millions of multiplies per second obtained from modern computers rely on "pipelining" many multiplies through the computer, or on using many multiplying units connected in an array, so that they can communicate. The current success of most large-scale simulations is heavily dependent on the fact that the method can exploit parallel computation with close to 100 per cent efficiency; usually the reason is that the similar calculations which have to be done at every site of the lattice of points in the simulation are perfectly handled by, for

example, a communicating array of processors.

The first QCD calculations on an array machine were done by the Edinburgh group on the ICL Distributed Array Processor (DAP). The potential of arrays of microprocessors, and their relative ease of construction has already encouraged physicists to build special purpose machines for scientific calculations: for QCD, a 64-processor machine built at the California Institute of Technology is already producing interesting results and at Columbia a project is nearing fruition to build a 64-mode machine a hundred times more powerful (and probably more powerful for this work than any available commercial machine). Companies such as Floating Point Systems, Cray and CDC are

responding with projects involving configurations of several communicating supercomputers, and Denelcor also have ambitious plans. VLSI chips, such as special-purpose arithmetic processors, the GEC GRID chip, and the Inmos transputer, with self-contained processor, memory and communications, will enable massively parallel multi-processors of enormous power to be built. Since these remarks do not even touch upon IBM or Japanese intentions, there can be no doubt that the impact of parallel processing in the next few years will result in an even more rapid growth in computer power than has been previously witnessed. □

D.J. Wallace is in the Department of P University of Edinburgh, EH9 3JZ, UK.

Enzyme therapy

Treatment of inborn errors of metabolism by transplantation

from Reed E. Pyeritz

FUNCTIONAL deficiency of a specific enzyme has been found in the majority of the more than 200 inborn errors of human metabolism. Potential treatments of the resulting clinical abnormalities are nearly as numerous as the different molecular pathologies underlying these enzymopathies. The paper by P.W. Gasper *et al.* on page 467 of this issue¹ illustrates and extends one promising approach — that of bone marrow transplantation.

Some forms of treatment have been impressively effective. For example, persons with one form of cystathionine synthase deficiency become biochemically normal when treated with pharmacological doses of pyridoxine; if detected and treated early in life, they may be spared all of the severe complications of homocystinuria. Similarly, restriction of dietary protein, in particular phenylalanine, markedly ameliorates the phenotype of phenylketonuria and results in nearly normal intelligence in children otherwise doomed to profound retardation. And the invariably lethal severe combined immunodeficiency produced by a lack of adenosine deaminase can be treated effectively by partial exchange transfusions because the enzyme is present in normal erythrocytes.

The last example differs from the others as the functional enzyme is administered from an external source. This has been attempted in the treatment of numerous inborn errors, but rarely with the success achieved in adenosine deaminase deficiency. Problems with enzyme therapy are legion. They include the need for a source of adequate enzyme, for purification of non-immunogenic and non-pyrogenic enzyme at reasonable cost and for delivery of the enzyme to appropriate tissues. Moreover, even when helpful, such treat-

ment is transient at best; it is limited by the life span of the organelle or cell carrying the enzyme and by the clearance and degradation of the enzyme, or by both. Furthermore, treatment of many conditions that produce neurological deterioration or mental retardation is limited by the blood-brain barrier, which prevents active enzyme from gaining access to the central nervous system².

For these reasons, investigators have been focusing on more permanent, and ultimately more physiological, methods of enzyme replacement therapy. For some, the final goal is either the addition of a functional copy of whichever mutant gene causes the condition, or the activation of another locus in the genome that can substitute, at least in part, for the mutant one. Although many technical problems remain — including those of targeting the functional gene to the appropriate tissue and then ensuring the proper physiological regulation of its transcription — gene therapy of somatic tissue has been attempted, will undoubtedly be successful in the near future, and may become the treatment of choice for some inborn errors before the end of the decade.

Transplantation of cells, tissue or whole organs affords another method of providing a patient with a constant, and sometimes regulatable, source of enzyme. In some cases, transplantation is effective in replacing an organ damaged by the metabolic defect. For example, a normal kidney transplanted to a patient with Fabry's syndrome has enough α -galactosidase activity to protect itself from the metabolic overload of neutral glycosphingolipids it encounters, but insufficient activity to treat the entire patient³. In other disorders, transplantation of a single organ may be

sufficient to improve markedly, or even cure, the major clinical effects. Liver transplantation in a variety of conditions, such as type I glycogen storage disease⁴, Wilson's disease and α -1 antitrypsin deficiency fall into that category.

Cells of the bone marrow offer several advantages in this regard. First, the techniques of bone marrow transplantation have become standardized, predictable and reasonably effective. Second, a variety of stem cells exist in marrow, each capable of performing different, if overlapping, metabolic activities. Third, mature cells from bone marrow precursors circulate to all organs of the body. Finally, enzymes of bone marrow-derived cells should be able to function in transit to reduce the toxic metabolites carried in the plasma, and hence their deposition in peripheral tissue, although the problem of the blood-brain barrier remains. Bone marrow transplantation has been notably successful in treating a number of heritable disorders, including those that are primarily erythropoietic (sickle cell disease⁵, thalassaemia⁶), immunological (various immunodeficiency states), reticuloendothelial (Gaucher's disease⁷), a combination thereof (Wiskott-Aldrich syndrome⁸), or osteoclastic (severe osteopetrosis⁹).

Lysosomal storage disorders, in which deficiency of a single enzyme leads to ineffective catabolism of macromolecules, have long been prototypes in the study of the therapy of genetic diseases. Patients with many of the mucopolysaccharidoses and sphingolipidoses have been treated over the past decade by all of the methods noted above, except gene replacement. Some encouraging, but preliminary, success with bone marrow transplantation has been reported in lysosomal disorders that lack primary central nervous system involvement, such as Gaucher's disease⁷ and Morquio's syndrome¹⁰. However, early reports of mental improvement in Hurler's syndrome¹¹ have largely been tempered by longer experience or the development of graft-versus-host disease.

Mucopolysaccharidosis VI (MPS VI; Maroteaux-Lamy syndrome) is the result of arylsulphatase B deficiency and results in severe skeletal, cardiac and pulmonary complications while sparing intellect. Bone marrow cells produce arylsulphatase B, so bone marrow transplantation could improve the condition or, if performed early enough in life, modulate the short stature, skeletal deformity, coarse facies and infiltration of the myocardium that inevitably occurs. To investigate the potential of such therapy, Gasper *et al.*¹ took advantage of an animal model for MPS VI: cats deficient in arylsulphatase B develop clinical features similar to humans. Soon after engraftment of bone marrow from an unaffected littermate, an arylsulphatase-deficient cat showed signs of biochemical improvement. Reduced urinary excretion of mucopolysaccharide metabolites and increased leukocyte

arylsulphatase B activity persisted for the length of the study, 250 days. The most objective sign of clinical improvement was a resolution of corneal clouding, although other signs, such as the cat's ability to walk and chew, its coarse facies and demeanor, also seemed to improve. It will be of interest to determine how much clinical improvement is possible in this and similarly-treated cats, and to document the pathological improvement at the end of their substantially lengthened lives.

Animal models for inborn errors of metabolism could lead the way to a much wider use of clinical marrow transplantation. Animals will be particularly valuable for testing methods of broaching

or circumventing the blood-brain barrier in those conditions with neurological sequelae¹². □

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Reed E. Pyeritz is in the Medical Genetics Clinic, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205, USA.

Immunology

T-cell receptor companions

from M. J. Owen

WITH the molecular analysis of the T-cell antigen receptor, molecular biologists have recently begun to solve the outstanding immunological question of how T cells recognize antigens and thus contribute towards the antigen-specific immune response. As already described in these columns¹, the receptor is composed of a β chain, which was well defined some months ago, and an α chain whose partial amino acid sequence^{2,3} has recently confirmed a tentative identification from cDNA clones^{4,5}. But the heterodimer formed by these two chains can, so far as is known, subserve only the recognition function of the molecule. Some other structure, therefore, must be responsible for transducing the signal to the interior of the cell and initiating the cellular response to antigen. An obvious candidate for this function is the so-called T3 molecule, which is physically associated with the receptor heterodimer⁶. T3 is a glycoprotein complex of three polypeptide chains (γ , δ and ξ). It, too, is beginning to yield to the all-embracing techniques of molecular biology as shown, in particular, by a paper from Terhorst's laboratory on page 413 of this issue⁷.

The T3 glycoprotein is unlikely to play a direct role in specific binding of antigen but is nonetheless probably obligatory for receptor function and may play a part in the stabilization of the initial interaction between receptor and antigens, as well as in signal transduction⁸. In this regard it is intriguing that calcium fluxes can be induced in T cells by antibodies against the T3 glycoprotein⁹. Furthermore, the expression of T3 is apparently linked to that of the antigen receptor, since in no case has one been detected at the T-cell surface in the absence of the other.

To clone the δ chain of T3, Terhorst's group has used the protein sequence route in which oligonucleotide primers are synthesized on the basis of a partial protein

sequence and used as probes with which to extract the desired cDNA species. (It is worth pointing out for the aficionados that the hybridisation probe used was a 19-mer and 15-mer mixture, consisting of 576 and 128 different sequences, respectively; this must represent a record for the redundancy of a primer used successfully in a cDNA cloning exercise.) The deduced primary sequence of the T3 δ chain confirms much of the biochemical data reported by Terhorst's group and by others, and contains few revelations or surprises. Predictably, it shows no sequence homology with members of the multi-gene family that includes the genes for the T-cell receptor, immunoglobulins and the major histocompatibility antigens. One point of interest is that the human δ -chain probe cross-hybridizes with a mRNA species in mouse T cells (as detected by Northern blotting analysis), which represents the first convincing demonstration of a murine equivalent of at least part of the T3 complex. The failure to identify a T3-like molecule on the surface of mouse T cells by means of serology has been a point of some concern to those who advocated a pivotal role for T3 in the T-cell response to antigen.

One possible clue to the mechanism of association of the α - β dimer with the T3 complex has emerged from this study. Terhorst's group point out that the transmembrane segments of the α and β chains of the structure both contain a lysine residue whereas an aspartic acid side chain is located in the corresponding region of the δ -chain transbilayer peptide. The ion pair that would form between these residues in the hydrophobic environment of the lipid bilayer would provide a strong stabilizing force for the T3-receptor complex. If this speculation is correct, *in vitro* mutagenesis experiments in which the transmembrane charged residues are altered should prove rewarding.

The minimal array of proteins involved

in the response to antigen on the surface of an effector T cell seems to be the T3-receptor complex, the T4 or T8 glycoproteins, and possibly the mysterious protein described by Tonegawa's group and originally thought to be an α chain candidate¹⁰. The availability of clones encoding all of these surface structures will be necessary before molecular immunologists can begin to manipulate the system and provide a complete molecular description of the recognition and activation processes. The isolation of a cDNA clone encoding the δ chain is an essential first step towards defining the role of T3. The partial protein sequence of the T3 ξ chain reported on page 455 of this issue¹¹ should provide the basis for the cloning of its cDNA. And the deduced protein sequence of this chain will prove especially interesting since it is postulated by Terhorst and his colleagues to be a constituent of a putative calcium channel involved in the process of activation of the T cell. At the current rate of progress, it should not be long before the sequence is reported and can be commented on in these columns. □

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M. J. Owen is in the ICRF Tumour Immunology Unit, Department of Zoology, University College London, London WC1E 6BT UK.



100 years ago

OVER-PRESSURE IN ELEMENTARY SCHOOLS

Take the typical case of a class of seventy children, starting with about the same attainments. The bulk of these will be average boys, or girls, as the case may be, fairly healthy and intelligent, not given to over-much study, but still ready to fall in with the requirements of the school. But there will also be some half-starved children who often come without any breakfast, dull children — descendants of a wholly uncultured race — feeble children, and others averse to any restraint and constitutionally irritable, together with children who are weary with toil at home. Besides these there are the exceptionally clever children, who are in danger of under-pressure and the over-sensitive or ambitious who are prone to over-work themselves. It is evident that the general scope of the instruction must be adapted to the average of the class.

From *Nature* **31**, 73; 27 November 1884.

Haematopoietic growth factors

SIR — May we add some important points to those made by T.M. Dexter in his News and Views article on haematopoietic growth factors¹. The development of blood cells requires a programme for the multiplication of stem cells and their differentiation to various types of mature cells with different functions. The mature cells no longer multiply. The normal developmental programme also has to include a mechanism for coupling the induction of multiplication in stem cells to the induction of differentiation, so as to maintain the normal balance between undifferentiated and mature cells. The points we wish to add are that multiplication (growth) and differentiation of blood cells are induced by different proteins, those that induce growth and those that induce differentiation, and that coupling between growth and differentiation can be achieved by a growth factor switching on production of a differentiation factor.

In cells of the myeloid series there are four different growth-inducing proteins. These are now called colony stimulating factors (CSF), or macrophage and granulocyte inducers — types 1 (MGI-1)¹⁻⁴. Of the four growth factors, one (M7) induces the development of clones with macrophages, another (G7) clones with granulocytes, the third (GM) clones with both macrophages and granulocytes, and the fourth (also called interleukin-3), clones with macrophages, granulocytes, eosinophils, mast cells, erythroid cells, or megakaryocytes. This multigene family represents a hierarchy of growth factors for different stages of blood cell development as the precursor cells become more restricted in their developmental programme. How do normal myeloid precursor cells induced to multiply by these growth factors develop into clones that contain mature differentiated cells that stop multiplying when they terminally differentiate? This has been answered by experiments that complement those discussed by Dexter¹.

Normal myeloid precursors require addition of one of the myeloid growth factors for cell viability and multiplication. But there are myeloid leukaemic cells that are viable and multiply without adding growth factor, and that can be induced to differentiate to non-malignant terminally differentiated macrophages or granulocytes by proteins which are different from the growth factors called CSF or MGI-1^{2,3}. These differentiation-inducing proteins, which also induce differentiation of normal myeloid cells^{2,3}, have been called MGI-2^{2,5} or differentiation factors (DF)^{6,7}. The injection of these MGI-2 proteins into leukaemic mice inhibits the development of myeloid leukaemia^{3,8}. Normal myeloid precursor cells cultured with one of the myeloid growth factors endogenously produce the differentiation factor MGI-2^{2,3,9} and this serves as an efficient mechanism to

couple growth and differentiation. Differences in the time of the switch-on of the differentiation factor can produce differences in the amount of multiplication before differentiation. There is more than one type of MGI-2^{5,10}. Different growth factors may switch on different differentiation factors and this may determine the differentiated cell type.

It has been suggested from experiments with WEHI-3B myeloid leukaemia cells that one of the myeloid growth factors (MGI-1G = G-CSF) can also act as a differentiation factor for these leukaemic cells⁴. This has not so far been found with other clones of myeloid leukaemic cells, and studies with these other clones have identified MGI-2 proteins that have no MGI-1G activity^{3,5-7}. In addition, MGI-2 but not MGI-1 binds to double stranded DNA¹¹. The form of MGI-1G used in the experiments with WEHI-3B may be a hybrid molecule, but there is also a different explanation. WEHI-3B cells constitutively produce their own growth factor and under appropriate conditions can be induced to endogenously produce their own differentiation factor¹¹. As with normal myeloid precursors, WEHI-3B cells may thus be induced to differentiate indirectly by the growth factor MGI-1G (G-CSF). In addition to these results with myeloid cells, different proteins that induce growth and differentiation have been found with B lymphocytes¹² and this presumably also applies to the other types of blood cells. Induction of differentiation factor may also play a role in the normal developmental programme of other cell types.

LEO SACHS
JOSEPH LOTEM

Department of Genetics,
Weizmann Institute of Science,
Rehovot 76100, Israel

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Calibrations for nuclear winter

SIR — In their recent complaint¹ about John Maddox's criticisms^{2,3} of their climate modelling work on the 'nuclear winter' concept⁴, Turco *et al.* attempt to buttress their case by stating that the climate model they used was partly calibrated by (1) 12 years of research on

martian dust storms, (2) the climatic consequences of volcanic explosions on Earth, and (3) the possible collision of an asteroid or cometary nucleus with Earth at the time of the Cretaceous/Tertiary extinctions. In addition, they say that their work was reviewed by a large number of experts and that it referred to many previous studies. If these reasons are indeed the basis for their confidence, the criticism they received was well justified.

To begin with, what good is a technique that is only *partly* calibrated? And how can a model be calibrated against a *possible* phenomenon which may or may not have actually occurred, and at that in the distant past? With respect to the climatic consequences of volcanic explosions, Turco *et al.* indicate in their complaint that these are caused primarily by sulphuric acid aerosols and not by the smoke and dust that is supposed to operate in the nuclear winter scenario. So how can this comparison be of any use? In addition, of what real comparative value is the planet Mars? It has no liquid water on its surface, while Earth is 70% covered by seas; and its atmospheric mass is miniscule. Dust there operates almost as if it were in a vacuum; and, again Turco *et al.* claim that it is not dust but rather sooty smoke from fires that is the major cause of nuclear winter. And as for citing lots of background material and getting the opinions of a large number of experts, what does that *prove*? Absolutely nothing, which is precisely the point made by John Maddox, and one which I heartily endorse.

SHERWOOD B. IDSO

Institute for Biospheric Research,
631 E. Laguna Drive,
Tempe, Arizona 85282, USA

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DNA methylation and G + C rich DNA

SIR — In a recent 'News and Views' article, Max collated evidence showing that the 5' region of certain genes is undermethylated in the cells of all tissues examined including sperm¹. This was used to explain the recent observations² that CG dinucleotides are present in unusually high amounts in certain regions of some genes. Where cytosine methylation does not occur, deamination of a cytosine base in DNA will produce uracil which can be removed from the DNA and the original sequence re-established. However, the occasional deamination of methylcytosine to thymine in DNA will lead, over many generations, to the loss of CG dinucleotides from the genome — so called CG suppression³. McClelland and Ivarie⁴ found a reduced suppression in the 5' flanking regions of some vertebrate genes which might imply that these regions are

undermethylated in the germ line. More recently Erickson⁵ has pointed out that several genes are highly methylated in sperm DNA, a finding which does not support the idea that in these cases a lack of methylation is the cause of the reduced CG suppression.

There are two ways to explain a lack of CG suppression in a particular region. As suggested by Max and explained above this would result if the CGs in that region are not methylated. It would also result if methyl CGs are not deaminated in that region and we have recently presented evidence to support this mechanism⁶. An analysis of various regions of vertebrate genomes demonstrates that CG dinucleotides occur at the expected frequency wherever G + C rich regions occur. (G + C rich regions having a composition of greater than 60% G + C.) The increased stability of the double helix in G + C rich regions will mitigate against the deamination reaction which requires the DNA to be single stranded. Even if heavily methylated, we would predict that CG dinucleotides will be maintained in these very G + C rich regions of DNA and that is what is found.

Deamination of methylcytosine in CG dinucleotides will lead to a decrease in the G + C content of DNA³ but where these dinucleotides are maintained no change in base composition would be expected. A + T rich regions should remain A + T rich and there is no relevant reason why they should become G + C rich. This implies that those regions which fail to show a suppression of CG dinucleotides have been rich in G + C for a very long time and this may be why they are resistant to deamination. Other events may lead G + C rich regions to mutate to a more average composition which may then allow methylcytosine deamination to occur, thereby accelerating the change to an A + T rich composition. Where a high G + C content is maintained or arises either by chance or because of selection it shows little CG suppression.

CG dinucleotides are highly suppressed in regions of the vertebrate genome containing less than 40% G + C and such regions have little remaining scope to exhibit further base changes as a result of methylcytosine deamination. Such mutations are expected to occur only in regions of intermediate composition where a number of methyl CG dinucleotides still remain in an environment conducive to deamination.

We do not argue against the proposal that lack of cytosine methylation may be the cause of the lack of CG suppression in some cases and indeed Cooper *et al.*⁷ have shown that short stretches of unmethylated DNA are present in the vertebrate genome. Nonetheless, we are of the opinion that a simple failure to deaminate methylcytosine in G + C rich regions may be a more general explanation for the absence of CG suppression in G + C rich regions of the vertebrate genome.

We thank Dr Max for his comments on this letter and Dr Bird for helpful discussions.

R. L. P. ADAMS
R. EASON

Department of Biochemistry,
University of Glasgow,
Glasgow G12 8QQ, UK

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Germ-line gene therapy a misnomer?

SIR — I would like to add to the medical and deontological reservations raised by M.E. Pembrey¹ regarding gene therapy. Two modes of gene therapy are considered: one involving intervention at the level of somatic cells on their immediate precursors (such as bone marrow cells²) and the second, modification of the germ line level³. The commonly implied advantage of germ-line gene therapy is the the correction of the defect could be made transmissible to further generations.

But, at present, there is no way to control or target the site of gene insertions, which seem to occur at random⁴. Consequently, deficient and inserted loci will, in most cases, lie on different chromosomes (or be separated by a large distance on the same chromosome) and so will segregate more or less independently. Thus, given a homozygous recessive defect, and as long as only a single chromosome can be the target for insertion, descendants will have only a 50% chance of inheriting the correcting locus along with the defective locus. That is, the so-called germ-line gene therapy will effectively control a genetic disease in the corrected individual, but the defective locus and potential for disease will still be transmitted to the progeny. Evidence is also accumulating demonstrating that this randomness of insertion bears a significant mutagenic potential⁵. Multiple independent insertions of the correcting locus would thus not be a solution to this problem, since although increasing the likelihood that offspring receive a "correcting" locus, they would also increase the likelihood of insertional mutagenesis.

Thus, at present, germ-line gene therapy of an individual would not eliminate the necessity to reiterate this therapy in his offspring, since diagnosis for the absence of the correcting gene in the offspring would have to be delayed to the embryo stage with present techniques — too late to apply germ-line gene therapy *de novo*. Arguments for germ-line intervention should thus take into consideration that the benefits are mostly restricted to one gener-

ation only. The possibility of insertional mutagenesis, in fact, seems to suggest that germ-line therapy is more likely to increase future genetic problems than it is to cure them.

JACQUES BECKMANN

Department of Plant Genetics
and Breeding,
The Agricultural Research Organization,
Bet-Dagan, 50-250,
Israel

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Three-dimensional chemical waves in metals

SIR — The review article by Winfree and Strogatz¹ mentions rotating spiral waves in a modified Belousov² chemical reagent, in a monolayer of *Dictyostelium discoideum* cells responding to a pulse of cyclic adenosine monophosphate³ and in successive photographs of the retina of a chicken⁴.

The photograph of a scroll wave in modified⁵ Belousov-Zhabotinsky reagent is reminiscent of the mechanism by which dislocations may multiply in metals first suggested by Frank and Read⁶. In this model, a line representing a dislocation in a three-dimensional dislocation network in a crystal is successively deformed by a shear stress so as to produce ring dislocations. An unlimited amount of slip may be produced.

The stress required for this process is of the order of Gb/l where G is the shear modulus, b is the Burgers vector, and l is the length of the line. Typically, for a metal $G = 500$ GPa, $b = 0.25$ nm and $l = 100$ μ m. This gives a stress of the order of 10^{-6} pascals and an energy of the order of 10^{-10} joules per metre of dislocation line. This corresponds to an energy of approximately 10 eV per atom along the dislocation line.

The value of 10 eV is in good agreement with the energy of a typical chemical bond and thus the initial dislocation line may be seen as an organizing centre for the generation of ring dislocations which are analogous to chemical waves travelling through the medium.

D.H. EVANS

Department of Applied Physics,
Sheffield City Polytechnic,
Pond Street,
Sheffield S1 1WB, UK

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Multiple specific contacts between a mammalian transcription factor and its cognate promoters

David Gidoni, William S. Dynan & Robert Tjian

Department of Biochemistry, University of California, Berkeley, California 94720, USA

The human transcription factor Sp1 binds upstream of certain viral and cellular promoters and activates initiation of RNA synthesis from these promoters by RNA polymerase II. The Sp1 binding sites of both simian virus 40 and a related monkey promoter contain multiple copies of the sequence GGGCGG, three or four of these sequences forming close contacts with Sp1. The clustered contacts fall on one strand of the DNA and are arranged similarly in the major groove of the DNA helix in both promoters.

MOST promoters for eukaryotic protein-coding genes contain distinctive upstream control sequences required for gene expression. Although the molecular mechanism by which these upstream sequences act to modulate transcription is not understood, it probably involves promoter-specific protein factors that interact with the DNA and influence the core transcription machinery to initiate RNA synthesis from particular sites.

Recently, two such promoter-specific transcription factors have been isolated and characterized: one, Sp1, interacting specifically with upstream control sequences of the simian virus 40 (SV40) promoter^{1,2} and a second, HSTF, involved in transcription of *Drosophila* heat shock genes³. In both cases the factors have been partially purified from cell or tissue extracts, their respective bind promoters and activate transcription *in vitro*. The stimulation of SV40 transcription by the Sp1 factor is dependent on sequences within a region of 21-base pair (bp) direct repeats located 48–103 bp upstream from the start sites for early transcription (ref. 2; D.G. and R.T., unpublished data). Sp1 binding activates transcription of both viral strands; sequences within the binding region are required for *in vivo* and *in vitro* transcription^{4–10}.

Because Sp1 is found in uninfected cells, it seems likely that it plays a role in the expression of cellular as well as viral genes. We have discovered recently a strong binding interaction between Sp1 and two binding sites occurring in a 450-bp region of the monkey genome (W.S.D. *et al.*, unpublished data). This putative monkey promoter sequence is homologous to sequences in the SV40 promoter region^{11,12}; it also directs transcription *in vivo* both in its normal chromosomal location and in an expression vector¹³.

A comparison of Sp1 binding regions in SV40 with those from the host cell should facilitate identification of specific features involved in binding and transcription. The boundaries of the SV40 and the two monkey Sp1 binding regions have been identified by DNase footprinting (ref. 2; W.S.D. *et al.*, unpublished data). The overall lengths of the regions range from 18 to 60 bp and each is associated with direct repeats of 14–21 bp. The prominence of the sequence 'GGGCGG' (the GC box) repeated many times in all three binding sites suggests that it has an important role in the recognition of the promoter region by Sp1. The ability of the footprinting technique to resolve specific features of the binding interaction is rather limited, however, in particular precluding identification of the nucleotide residues within the protected region likely to make specific contacts with the protein.

In the present experiments, we have used dimethyl sulphate to probe the binding of Sp1 to specific sites in both the SV40 and monkey genomic sequences. Dimethyl sulphate is a reactive electrophile methylating the N-3 positions of adenine and the

N-7 positions of guanine in duplex DNA¹⁴. Because dimethyl sulphate is a small molecule, protection from methylation can be used to infer the location of specific A or G residues forming intimate contacts with sequence-specific binding proteins¹⁵. By observing the pattern of dimethyl sulphate protection in the presence of Sp1, we have identified the particular GC boxes that appear to make close contact with this factor and have constructed a model for the internal structure of the binding sites.

Sp1 binding to SV40 promoter

We have shown previously that a fraction containing Sp1 from cultured human cell extracts is capable of protecting a site in the SV40 promoter region from DNase attack². In those experiments, a duplex DNA fragment was labelled at one end, incubated with Sp1 and subjected to limited cleavage with DNase I. The presence of specifically-bound Sp1 results in a gap, or footprint, in the ladder of cleavage products revealed by electrophoresis. The footprint is seen on both strands and is suppressed in the presence of excess unlabelled SV40 DNA². Partial or full protection extends ~60 bp (nucleotides 45–104), overlapping the 21-bp repeated elements and containing GC boxes II–VI and a portion of I (Fig. 1a).

To obtain a more detailed picture of the binding interaction between Sp1 and the SV40 sequences, we performed dimethyl sulphate methylation protection experiments (Fig. 1b). Sp1–DNA complexes were allowed to form at 0 °C, then subjected to partial methylation with dimethyl sulphate as described in Fig. 1 legend. Methylated guanine residues were identified by cleavage of the DNA backbone¹⁵. In the presence of Sp1, 13 guanine residues on the late RNA sense strand (see ref. 16) of the SV40 control region showed specific and almost complete protection from methylation by dimethyl sulphate. These residues fall into three clusters corresponding to GC boxes III, V and VI. An additional four residues contained within the second GC box proximal to the sites of early transcription (GC box II) were weakly protected from methylation by Sp1 binding and there was also very slight protection in GC boxes I and IV. In addition, several guanine residues located near the clusters of protected residues were hypermethylated, perhaps because the local environment of bound Sp1 favours the methylation reaction.

In order to identify adenine as well as guanine residues that may be protected from methylation, Sp1 was incubated with DNA and treated with dimethyl sulphate as before, but the conditions of subsequent treatment were such that DNA was cleaved at both the N-7 methyl guanine and the N-3 methyl adenine¹⁵. In contrast to the pattern of protection seen with late-strand guanine residues, there was no significant methyla-

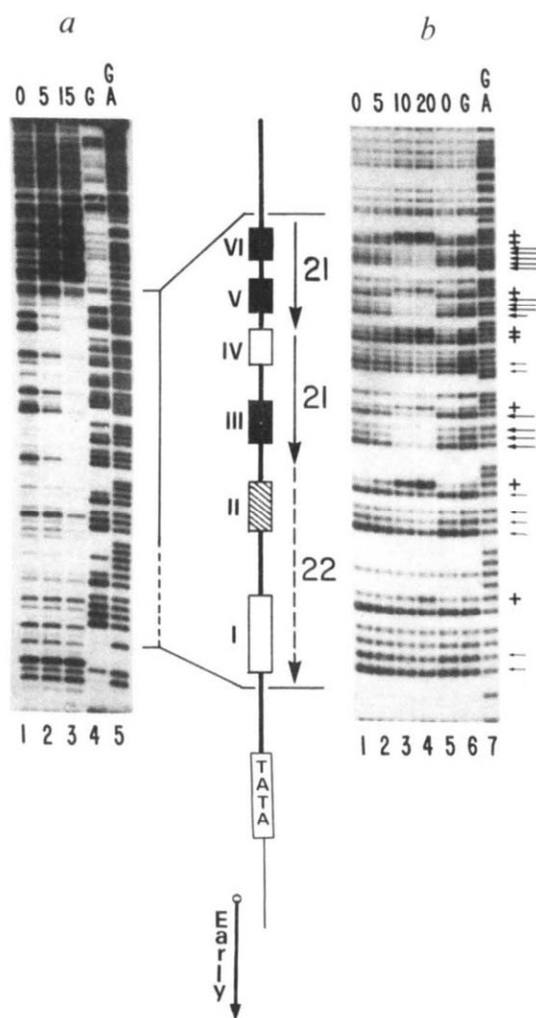


Fig. 1 Sp1 binding to the SV40 promoter region. *a*, DNase I footprinting. Reactions contained 0, 5 or 15 μ l of Sp1 as indicated. Brackets mark the limits of the protected region. Lanes G and G + A contain Maxam-Gilbert²⁰ sequence ladders prepared from the same fragment used for the binding. *b*, Dimethyl sulphate methylation protection. Reactions contained 0, 5, 10 or 20 μ l of Sp1. Arrows, protected residues; +, hypermethylated guanine residues. The central diagram schematically represents the Sp1 binding region. Rectangles, the six GGGCGG motifs in the SV40 21-bp repeat region. Filled, hatched and open rectangles represent strong, partial and weak protection from dimethyl sulphate, respectively. The 'TATA' box and direction of early transcription are also indicated.

Methods: Sp1 was partially purified by fractionation of a human (HeLa) whole-cell extract as described previously¹. The end-labelled DNA probe for binding experiments was prepared from the plasmid pX8, containing the SV40 genome with a *Xho*I linker inserted at the *Bgl*II site in the origin of viral replication⁵. The plasmid was digested with *Xho*I, labelled with [γ -³²P]ATP and polynucleotide kinase and digested again with *Pvu*II. The fragment containing the Sp1 binding site was gel-purified; this fragment is singly 5' end-labelled at a site downstream from the early 'TATA' sequence. In each binding reaction, 3 ng (17 fmol) of labelled probe fragment and 1,000 ng of unlabelled sonicated calf thymus DNA were incubated with 0–20 μ l Sp1 (0–10 ng total protein) for 15 min at 0°C in 50 μ l of the reaction buffer described by Dynan and Tjian². For DNase footprinting²¹ the reaction was warmed rapidly to room temperature and the DNA cleaved as described previously². For dimethyl sulphate methylation protection¹⁵, 1–4 μ l dimethyl sulphate was added and incubation continued for 1 min at 0°C. The reaction was terminated and DNA subjected to cleavage at sites of N-7 guanine methylation (described by Maxam and Gilbert²⁰). All samples were analysed on a denaturing 8% polyacrylamide sequencing gel.

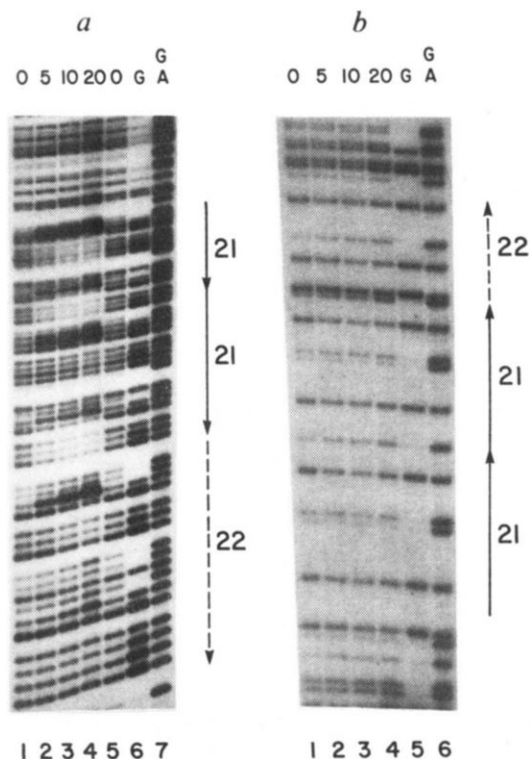


Fig. 2 Comparison of the dimethyl sulphate methylation protection pattern on opposite strands of DNA in the SV40 early promoter region. *a*, DNA probe 5'-end-labelled on the *Xho*I site downstream from the early 'TATA' box, prepared as described in Fig. 1 legend. *b*, DNA probe 5'-end-labelled at an *Eco*RI site upstream from the early promoter, prepared from the plasmid pSV07 as described by Dynan and Tjian². In both experiments, end-labelled DNA probe and unlabelled carrier DNA were incubated with 0–20 μ l Sp1 and treated with dimethyl sulphate as described in Fig. 1 legend. Simultaneous cleavage of N-7 methylated guanine and N-3 methylated adenine residues was carried out according to Gilbert *et al.*¹⁵. The Maxam-Gilbert G and G + A sequence ladders are shown alongside the dimethyl-sulphate protection pattern. Arrows, positions of the 21-bp repeats and the 22-bp sequences of the SV40 promoter.

tion protection or enhancement observed with adenine residues (Fig. 2*a*). No protection of either guanine or adenine was seen with an early-strand probe (Fig. 2*b*). Thus, Sp1 methylation protection appears to be limited to moieties in the major groove (N-7 of guanine but not N-3 of adenine) and to one strand of the DNA.

Sp1 binding to monkey promoter

The presence of Sp1 in uninfected cells suggests that a class of SV40-like cellular promoters may also be under the control of this transcription factor. We have recently characterized transcription and Sp1 binding properties of a cloned genomic monkey DNA sequence that exhibits *in vivo* and *in vitro* promoter activity (ref. 13 and W.S.D. *et al.*, unpublished data). DNase footprint analysis of the monkey DNA revealed two distinct and separate binding regions for Sp1, designated α and β (see map in Fig. 3). Sequence analysis of these two regions indicates that both contain multiple GC boxes arranged similarly to the SV40 21-bp repeat region.

We chose to focus on the β region for dimethyl sulphate methylation protection experiments both because it is located between divergent *in vivo* transcriptional start sites and because preliminary experiments showed a more distinct pattern of protection than in the α region. Side-by-side DNase footprinting and dimethyl sulphate methylation protection experiments are shown in Fig. 3*a* and *b*. The pattern of protected guanine residues in the monkey DNA is very similar to the SV40 promoter. Three of the six GC boxes on one strand of the duplex binding site were almost completely protected by Sp1 from

Fig. 3 Sp1 binding to the putative monkey promoter. **a**, DNase footprinting. Probe DNA was 5' end-labelled at a linker adjacent to an *Sph*I site located to the right of the β binding region. Brackets, region protected by Sp1. **b**, Dimethyl sulphate methylation protection using the same probe as **a**. DNA was cleaved at methylated guanine residues. Arrows, protected guanine residues; +, hypermethylated guanine residues. A schematic representation of the protected guanine clusters within the Sp1- β binding region is shown alongside **a** and **b**. The six GC boxes are represented by solid or open rectangles, indicating residues strongly protected from dimethyl sulphate or not protected, respectively. **c**, Dimethyl sulphate methylation protection of the opposite strand. Probe DNA was 5' end-labelled at the *Ava*I site to the left of the Sp1 binding regions. DNA was cleaved simultaneously at methylated guanine and adenine residues. **a-c**, Binding reactions and cleavage procedures as described in Fig. 1 legend. **d**, The two distinct binding regions for Sp1, α and β , detected by DNase footprinting (W.S.D. *et al.*, unpublished data). Filled rectangles, the GGGCGG sequence motifs in each binding region. Arrows, the 17-bp repeats and the E ('early') and L ('late') transcription directions. DNA probes in these binding experiments were prepared from the plasmid p7.02, provided by Drs J. Saffer and M. Singer.

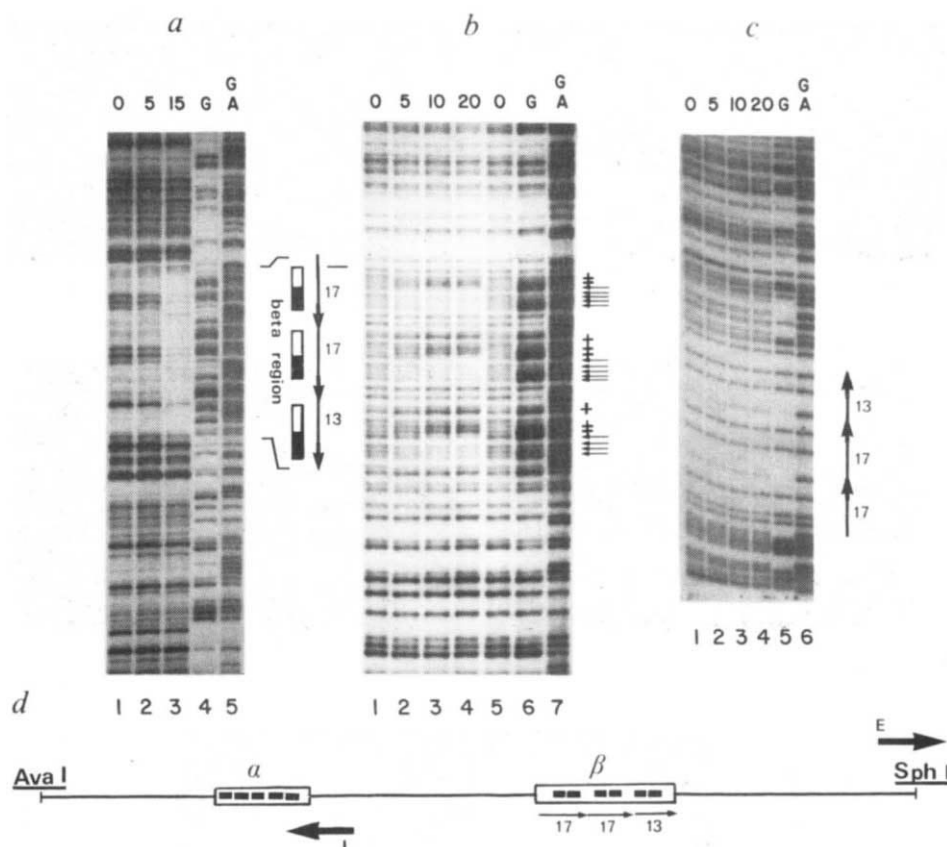
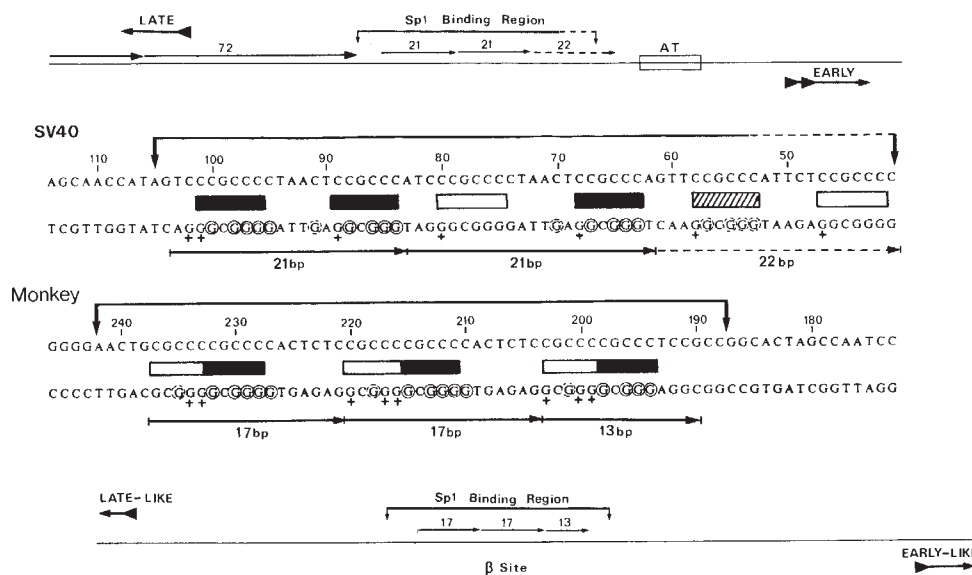


Fig. 4 Close contact points between Sp1 and SV40 or the cellular monkey promoter sequences. The sequences of SV40 and monkey DNA containing the Sp1 binding regions are displayed. Brackets, footprints of Sp1 within the viral and cellular promoters; circles, specific guanine residues protected from dimethyl sulphate methylation by Sp1 binding; dashed circles, partially protected residues; +, hypermethylated residues. Rectangles represent GC boxes containing unprotected (open), partially protected (hatched) and fully protected (solid) clusters of guanine residues. Arrows, direction of early (E) and late (L) transcription. The nucleotides of the SV40 sequences are numbered according to Tooze¹⁶ and those of the monkey sequences according to Queen *et al.*¹². Schematic diagrams of the transcriptional control regions of SV40 (top) and the monkey cellular DNA (bottom) are illustrated. The diagrams show the Sp1 binding sites and the relative positions of the bidirectional transcription initiation sites.



dimethyl sulphate attack (Fig. 3b) and hypermethylated guanine residues were found at positions directly adjacent to the methylation protection sites. No adenine residues were hypermethylated or protected on this strand (data not shown), nor was there any perturbation of methylation at guanine or adenine on the opposite strand (Fig. 3c).

Sp1 contacts on DNA helix

Data from the viral and cellular binding regions together suggest that Sp1 forms close contacts with only a selected group of guanine residues within the GC boxes. The protected atoms lie within the DNase I footprints and are all on one strand of the

duplex DNA (Fig. 4). No adenine residues are protected from modification, indicating that the contact points are located in the major groove of the double helical binding region. Although the precise nucleotide sequence and the spacing of the GC boxes are not identical in the viral and cellular promoter regions, the general pattern of close contact residues follows a similar spatial arrangement.

We have visualized the arrangement of Sp1-DNA close contact points in computer-generated stereoscopic images (Fig. 5). Each cluster of protected atoms occupies one half-turn of the DNA helix. Although protein structure cannot be predicted from methylation protection data alone, this elongated pattern of

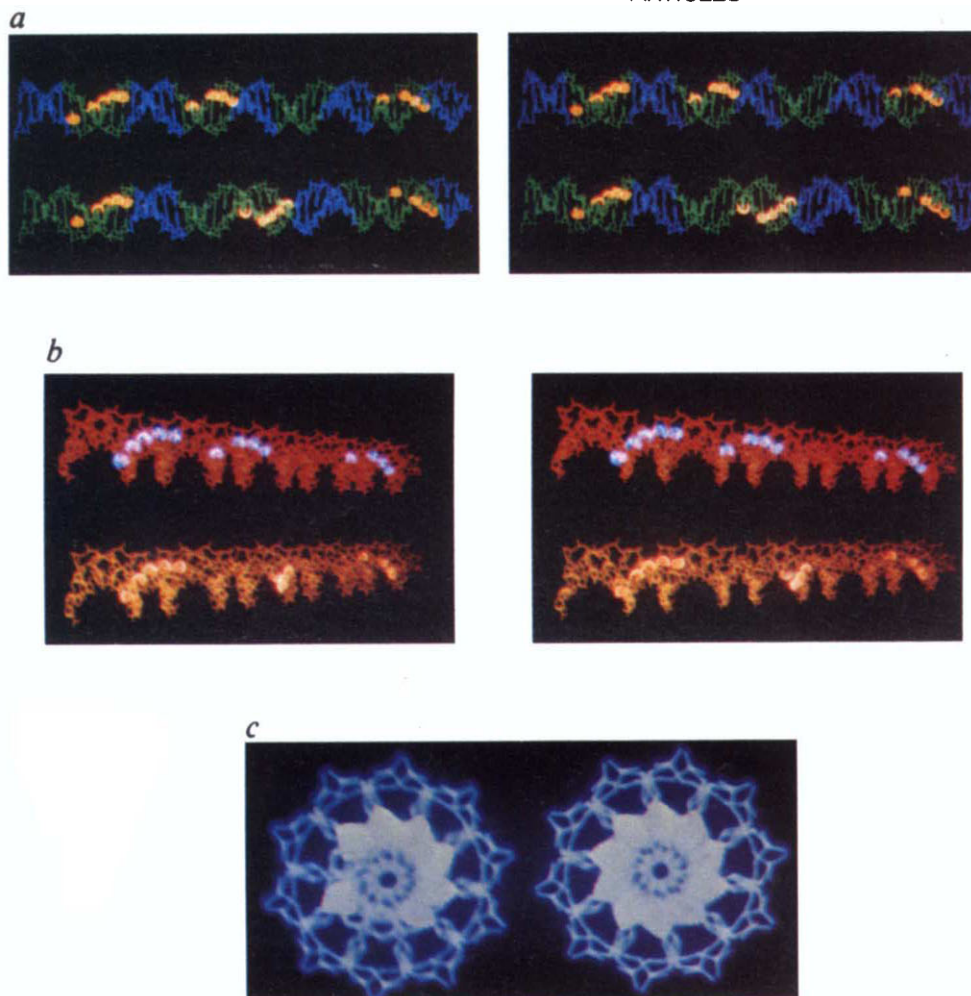


Fig. 5 Stereoscopic representations of Sp1 binding regions within SV40 and the cellular monkey promoter sequences. The two Sp1 binding regions have been aligned for maximum homology. *a*, Side view of SV40 (top helix) and monkey (bottom helix) binding sequences. The DNA sequences projected correspond to the approximate limits of the DNase footprint region. The GGGCGG sequences are highlighted in green and the surrounding DNA sequences represented in blue. The yellow spheres designate the van der Waals' radii of the guanine N-7 residues in close contact with Sp1 and strongly protected from methylation. *b*, SV40 (red helix) and monkey (orange helix) Sp1 binding regions viewed at an oblique angle to accentuate the location of close contact points within the major groove of the DNA helix. *c*, SV40 (left) and monkey (right) binding sequences are viewed down the axis of the helix. The positions of contact points with Sp1 are shown as white spheres around the DNA axis. The Sp1-DNA contacts were projected in three dimensions with the assistance of Dr Judith McClarin at the Computer Graphics Laboratory (University of California, San Francisco) as described by Jones and Tjian²².

protection is consistent with that expected if a rigid rod were laid in the major groove parallel to the DNA strands. This model is similar to that proposed for certain prokaryotic sequence-specific DNA-binding proteins, such as phage repressors, where an α -helical protein domain is believed to lie across the DNA in the major groove (see ref. 17 for review). Sp1 differs from these other proteins, however, in that only one strand of the DNA shows evidence of protein contact.

There is an interesting difference in the internal geometry of the Sp1 binding regions in SV40 and monkey DNA. In SV40, the protected residues in all three clusters are located predominantly on one face of the helix (Fig. 5*a*, top). By contrast, because of a 6-bp difference in the spacing, the centre contact cluster in the monkey β binding region is rotated relative to SV40, such that these residues are now on the bottom face of the helix. This difference in geometry can be seen readily in Fig. 5*b* and *c*. Interestingly, because of redundancy in the monkey sequence, alternate contacts are available at the top of the helix in a position fully homologous to those in SV40, but apparently are not used. This preference could arise from steric considerations as bulky binding moieties may not be accommodated easily in the restricted space between protected clusters. As a consequence of these possible steric interactions, the DNA helix may be strained in SV40 but not in monkey DNA. There is some experimental evidence to support this view. Our previous studies have shown the presence of several specific sites of enhanced DNase I cleavage in sequences just outside the footprint region in SV40, whereas such sites are not found in sequences flanking the monkey β Sp1 binding region (ref. 2; W.S.D. *et al.*, unpublished data).

Our results confirm the notion that structural moieties within the repeated GC boxes make a significant contribution to the sequence-specific binding of Sp1. However, one of the more striking findings from the dimethyl sulphate protection experi-

ments is that the GC boxes are markedly nonequivalent. This implies either that sequences outside the boxes are important in determining the course of binding, or that the placement of the boxes with respect to each other favours certain interactions at the expense of others. It is interesting to note that GC box IV, which lies in the middle of the SV40 binding region but is not protected from dimethyl sulphate, has been shown previously to contain a site hypersensitive to DNase I cleavage².

Strand specificity and multiple binding sites

The fact that we have observed binding only to one DNA strand may reflect bias inherent in our technique, because the reactive moiety we have examined in the major groove is found only in guanine and because the late-sense strand of the DNA is significantly more guanine-rich than the early-sense strand. However, the one guanine residue in the early strand of each GC box was never protected from methylation. This degree of strand specificity has not been observed with most prokaryotic DNA binding proteins, although there is some precedent among other eukaryotic proteins. Transcription factor IIIA, for example, which binds to and activates the genes for 5S RNA, makes contact primarily with the non-template strand of the DNA¹⁸.

Previous observations have shown that Sp1 binds to an extended and internally repetitious region in both the viral and cellular DNAs. The overall length of the DNase footprint can be as little as 18 bp of complete protection in the monkey α site and as much as 55 bp in the monkey β site (ref. 2; W.S.O. *et al.*, unpublished data). In addition to this length heterogeneity, the dimethyl sulphate protection experiments indicate that the internal structure of the binding region varies also. In particular, the rotation of the central contacts in the β site relative to SV40 suggests a degree of flexibility in the final conformation of the Sp1-DNA complex.

These findings support the notion that some binding regions may accommodate multiple protomers of Sp1, with each protomer interacting independently with a subset of sequences in the region. The existence of such individual sites can be tested using mutants altered in single GC boxes or variants naturally containing only one or two GC boxes. We have begun to analyse a set of such DNA templates, some of which were generated by oligonucleotide site-directed mutagenesis of the Sp1 binding region in SV40. Preliminary results support the multi-site model (D.G., H. Barerra-Saldana, K. Takahashi, P. Chambon and R.T., unpublished results; K. A. Jones and R.T., unpublished results; W.S.D. *et al.*, unpublished results).

Sp1 binding and transcriptional activation

Unlike the recognition sites for prokaryotic transcription factors such as phage repressors or the cyclic AMP receptor proteins, the Sp1 binding regions contain no sequence palindromes and are markedly asymmetric in base composition. Despite this asymmetry, there is good evidence that the Sp1-dependent transcription of SV40 occurs on both strands of the DNA. Addition of Sp1 to an *in vitro* transcription reaction results in the appearance of two transcripts that initiate on opposite strands at locations approximately equidistant from the centre of the binding region¹. Inversion of the SV40 21-bp repeats results in a promoter remaining competent to direct viral RNA synthesis in the early direction *in vivo*⁷.

We are thus left with a paradox in which binding of a factor

to an asymmetric sequence results in symmetric activation of transcription. If activation proceeds by protein-protein contact, similar protein recognition sites would then occur at either end of an asymmetric molecule. If, however, the Sp1 effect proceeds via a perturbation of the helix, one might expect not only that the effect on transcription could propagate in both directions, but also that some variation could be tolerated in the distance between the binding site and the start of transcription. In support of this idea, alteration of sequences in the 21-bp repeats has been reported to affect late transcription from sites as far away as 200 bp^{5,9,10}.

There is no direct evidence that Sp1 is bound to DNA *in vivo*. In one recent DNase hypersensitivity study, however, SV40 minichromosomes appeared to have a small protected region in the 21-bp repeats, flanked by regions of hypersensitivity extending to either side¹⁹. Final proof of the *in vivo* role of Sp1, as well as a more complete understanding of the nature of the binding interaction, awaits identification of the active molecule and development of improved techniques for measuring its interaction with the chromatin template *in situ*.

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Isolation of cDNA clones encoding the 20K T3 glycoprotein of human T-cell receptor complex

Peter van den Elsen, Beth-Ann Shepley, Jannie Borst, John E. Coligan*, Alexander F. Markham†, Stuart Orkin‡ & Cox Terhorst

Laboratory of Molecular Immunology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts 02115, USA

* Laboratory of Immunogenetics, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20205, USA

† ICI Pharmaceuticals Division, Mereside, Alderly Park, Macclesfield, Cheshire SK10 4TY, UK

‡ Department of Hematology, Children's Hospital Medical Center, Boston, Massachusetts 02115, USA

The complete amino acid sequence of one of the polypeptide chains of the human T3/T-cell receptor complex, the T3 glycoprotein (T3- δ -chain) of relative molecular mass 20,000, was deduced from cDNA clones derived from HPB-ALL cells and a human T-cell clone. Inspection of the 171-amino acid sequence reveals a signal peptide, a 79-amino acid extracellular domain, one transmembrane region and a 44-amino acid intracellular domain which show no sequence homology with members of the T-cell receptor/immunoglobulin/MHC multi-gene family. The T3 δ -chain is coded for by a single-copy gene the expression of which is restricted to T lymphocytes in humans and mice.

T LYMPHOCYTES have a central role in immune responses, eliminating cells that appear to be foreign and regulating the activities of other immune cells, including the antibody-producing B cells. Specific receptors are involved in antigen recognition by T-lymphocyte clones. Recently, it has been found that in humans and mice these T-cell receptors are sulphhydryl-linked heterodimers, which consist of two glycosylated polypep-

tide chains (α - and β -chains) of relative molecular mass (M_r) 37,000-50,000 (37K-50K)¹⁻³. In addition, genes encoding both the α - and β -chain have been isolated⁴⁻⁷. On the surface of human lymphocytes, the clone-specific (clonotypic) α - and β -chains of the T-cell receptor are associated with the three invariable T3 proteins, forming the so-called T3/T-cell receptor complex⁸⁻¹⁰. These T3 polypeptide chains have M_r of 25K (T3

Fig. 1 *a*, Oligonucleotide mixtures used as probes for cloning T3 δ -chain cDNA. Two regions of amino acid sequences from the N-terminal part of the T3 δ -chain were selected for synthesis of oligonucleotides. Probe 1 contained 576 different 19-mers with nucleotide sequences complementary to amino acid residues 2–8. Probe 2 contained 128 different 15-mers predicted from amino acid residues 12–16. Probe 1 represents (+)strand DNA and probe 2 (–)strand DNA. The oligonucleotide mixtures were chemically synthesized by the solid-phase phosphotriester method on an inorganic solid support and purified by HPLC as described previously^{34,35}. *b*, Result of second-strand synthesis using the 19-mer oligonucleotide mixture as primer. The ~0.62-kb cDNA product that is bracketed was used to generate the DNA library. O, Origin; XCF, xylene cyanole FF.

Methods: Total mRNA was extracted from HPB-ALL cells by lysis in a buffer containing 6 M guanidine hydrochloride³⁶. Poly(A)⁺ RNA was isolated by selective retention to oligo(dT) cellulose^{37,38}. 2 μ g of poly(A)⁺ RNA were converted to cDNA in the presence of 50 mM Tris-HCl pH 8.3, 1 mM each of the deoxynucleotide triphosphates (dNTPs), 50 mM KCl, 10 mM MgCl₂, 10 mM dithiothreitol, 18 μ g ml⁻¹ of oligo(dT)₁₂₋₁₈, 40 U placental RNase inhibitor and 400 U ml⁻¹ reverse transcriptase (Life Sciences Inc.) in a final volume of 100 μ l. Incubation was carried out for 90 min at 42 °C. The reaction was stopped by the addition of EDTA to a final concentration of 20 mM and the mRNA template was removed by alkaline hydrolysis³⁸. After neutralization and phenol extraction, unincorporated dNTPs were removed by gel filtration through a 1-ml Sephadex G-50 spin column³⁸.

0.375 μ g of oligonucleotide probe 1 to be used as primer was 5'-end labelled with 100 μ Ci of [γ -³²P]ATP (5,000 Ci mmol⁻¹, ICN Radiochemicals) and 20 U T4 polynucleotide kinase (BRL). The reaction was stopped by adding EDTA to a final concentration of 20 mM. Following phenol extraction, the 5'-labelled oligonucleotide mixture was added to the cDNA and both were recovered by ethanol precipitation in the presence of 0.3 M NaAc, pH 5.5. The pellet was washed once in 70% ethanol and resuspended in 67 μ l of 104 mM KCl, 45 mM Tris-HCl pH 7.5 and 6 mM MgCl₂. The primer was annealed to the cDNA by heating for 5 min at 68 °C followed by slow cooling to room temperature. Before second-strand synthesis, the mixture was kept in ice for 1 h. Second-strand synthesis was initiated by the addition of dNTPs to a final concentration of 0.5 mM, 2-mercaptoethanol to a final concentration of 0.5 mM and 10 U DNA polymerase I (Klenow fraction). This mixture, in a final volume of 100 μ l, was incubated for 20 h at 15 °C. 20 U reverse transcriptase was added and incubation was continued for 1 h at 37 °C. The enzymes were inactivated by the addition of EDTA to a final concentration of 20 mM. Proteins were removed by phenol extraction and double-stranded (ds) cDNA was recovered by ethanol precipitation following gel-filtration through a 1-ml Sephadex G-50 spin column. The termini of the ds cDNA were trimmed with 30 U ml⁻¹ of S₁ nuclease in the presence of 0.3 M NaCl, 0.03 M NaAc pH 4.5 and 10 mM ZnCl₂ for 30 min at 37 °C in a final volume of 100 μ l. Following phenol extraction and ethanol precipitation, the cDNA was size-fractionated in a 5% polyacrylamide gel and a specific product of ~0.6 kb was detected after autoradiography. This product was excised from the gel and DNA was isolated and purified as described by Maxam and Gilbert²³. Homopolymeric dC tails were added using 20 U of terminal transferase (BRL) in the presence of 0.6 M sodium cacodylate pH 7.19, 0.5 mM dithiothreitol, 2.5 mM CoCl₂, 0.5 mg ml⁻¹ bovine serum albumin (BRL) and 5 μ M dCTP in a final volume of 50 μ l for 20 min at room temperature³⁹. After enzyme inactivation and phenol extraction, ds cDNA was recovered by ethanol precipitation, annealed to *Pst*I-digested, dGMP-tailed plasmid vector pBR322 and used to transform *E. coli* MC1061 (ref. 40) to tetracycline resistance. A selected cDNA library of ~7,000 members was generated by plating the cells directly onto nitrocellulose³⁵. After replica-plating and amplification, the DNA was denatured and fixed onto the filters as described elsewhere³⁸. The filters were hybridized overnight at 36 °C (probe 1) or 26 °C (probe 2) in 6 $\times$ SSC. (1 $\times$ SSC = 0.15 M sodium chloride and 0.015 M sodium citrate). 1 $\times$ Denhardt's solution (0.02% each of Ficoll 400, bovine serum albumin and polyvinylpyrrolidone), 0.05% sodium pyrophosphate, 100 μ g ml⁻¹ of yeast tRNA and 10⁷ c.p.m. ml⁻¹ of 5'-³²P-labelled oligonucleotide probe 1 or 2 (specific activity ~4 $\times$ 10⁸ c.p.m. μ g⁻¹). Filters were washed extensively at room temperature in 6 $\times$ SSC plus 0.05% sodium pyrophosphate followed by stringent washes at 44 °C (probe 1) or 34 °C (probe 2) in the same buffer. The filters were exposed to Kodak XAR-5 film at -70 °C using an intensifying screen. One cDNA clone (pGBC-1) with an insert of 0.62 kb which hybridized to both probes was identified.

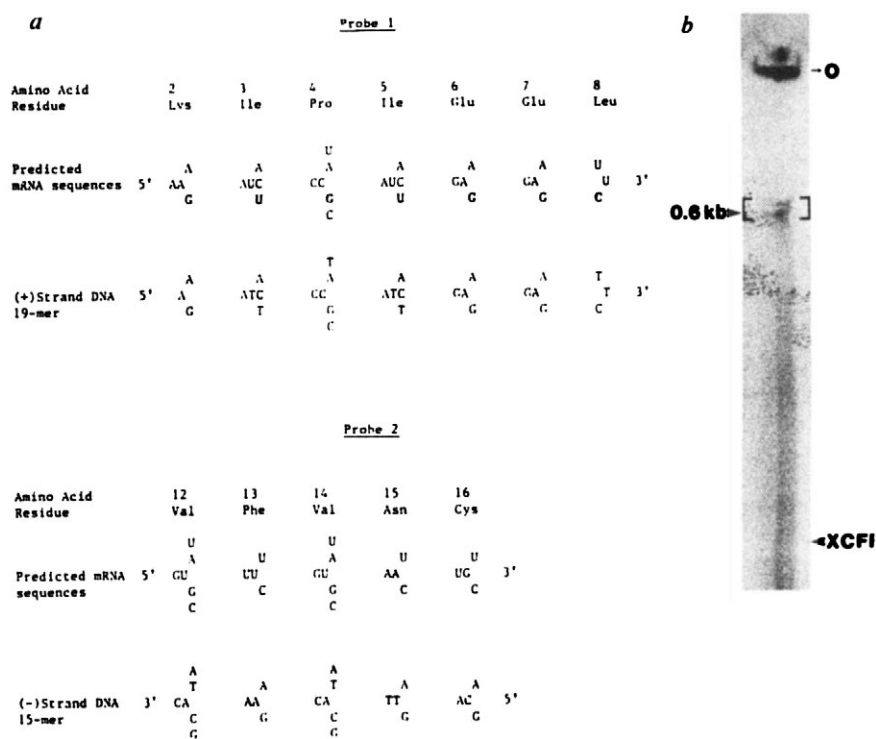
γ -chain) and 20K (T3 δ - and ϵ -chains). The T3 γ - and T3 δ -chains are glycoproteins whereas the T3 ϵ -chain contains no detectable oligosaccharides^{8,11-14}. The three T3 polypeptide chains are structurally unrelated to each other as determined by peptide mapping, N-terminal amino acid sequencing and their reactivity with an antiserum prepared using a synthetic peptide representing the 14 N-terminal amino acids of the T3 δ -chain¹⁵.

The T3 δ -chain is of particular interest because a monoclonal antibody that binds specifically to this glycoprotein is mitogenic for peripheral blood T lymphocytes¹⁶. This property had been ascribed earlier to other anti-T3 monoclonal antibodies, the exact target structure of which was unknown¹⁷⁻²⁰. When isolated from the plasma membrane, the T3 δ -chain has a polypeptide backbone of 14K after removal of N-linked oligosaccharides with endo- β -N-acetylglucosaminidase F (Endo-F)^{8,14}. In pulse-chase experiments using ³⁵S-L-methionine and ³⁵S-L-cysteine, a 23K intermediate was detected which was sensitive to endoglycosidase H (Endo-H) and Endo-F and which had a polypeptide backbone of 16K⁸. The N-terminal amino acid sequences of the 23K and 16K intermediates were identical to that of the 14K T3 (ref. 15). These observations indicated that both oligosaccharide and proteolytic processing might take place during the maturation of the T3 δ -chain. An alternative interpretation which postulated that the 16K product was an intermediate in the deglycosylation experiment could not be excluded, however.

Here we describe the isolation and characterization of cDNA clones that encode the complete amino acid sequence for the T3 δ -chain. The predicted *M_r* of the T3 δ -polypeptide chain is 16,719. This was confirmed by analysis on SDS-polyacrylamide gel electrophoresis of an *in vitro* translation product after hybrid selection. Taken together with our previous observations, these data provide strong evidence that proteolytic processing occurs at the C-terminus of the T3 δ -chain. Further inspection of the amino acid sequence revealed an 79-amino acid extracellular domain and a 44-amino acid intracellular domain separated by a transmembrane region of 27 amino acids. The amino acid sequence of this T3 chain shows no sequence homology with T-cell receptor α - and β -chains, immunoglobulins or T-cell surface structure Thy-1. T3 δ -chain mRNA was detected only in human haematopoietic cells of T-lineage. In murine helper T lymphocytes, a mRNA of similar size has been detected.

Isolation of cDNA clones

Plasmids with inserts coding for the T3 δ -chain were isolated from complementary DNA libraries using synthetic oligonucleotide mixtures as hybridization probes. From the available N-terminal amino acid sequence¹⁵, a 19-mer and a 15-mer oligonucleotide mixture was synthesized consisting respectively of 576 and 128 different sequences (Fig. 1*a*). First, in order to enrich for T3 δ -chain-specific sequences by size selection and to ascertain the presence of N-terminal protein coding sequences in the



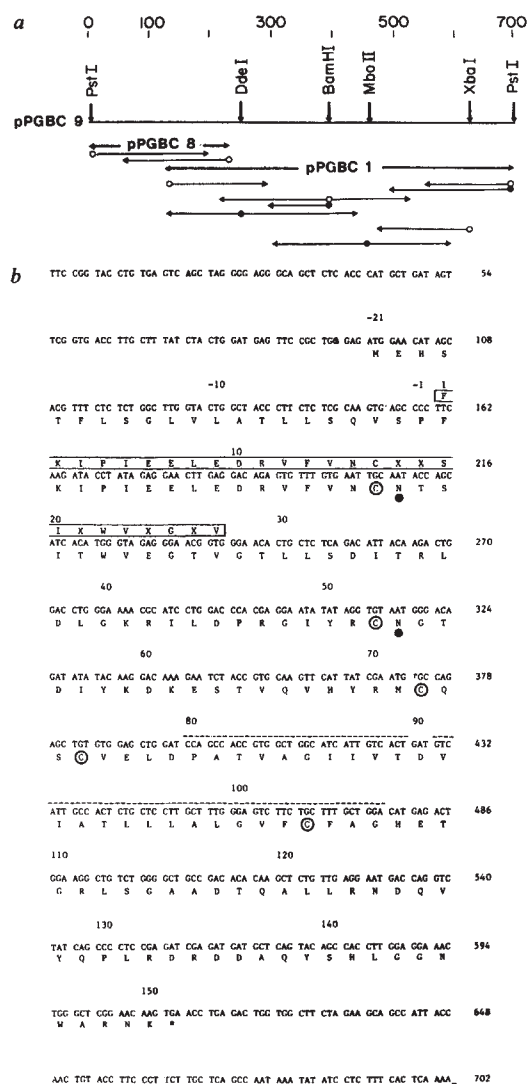


Fig. 2 Nucleotide sequence and predicted amino acid sequence of the T3 δ -chain. *a*, Partial restriction map of the T3 δ -chain and strategy for sequencing of clones pPGBC-1, pPGBC-8 and pPGBC-9. *DdeI*, *BamHI*, *MboII*, *XbaI* and the *PstI* cloning sites were 3'-labelled with either terminal transferase and [α - 32 P]dATP (NEN) or DNA polymerase I (Klenow fraction) and [α - 32 P]dNTPs (ICN) and 5'-labelled with T4 polynucleotide kinase and [γ - 32 P]-ATP. One-end-labelled fragments were generated either by cleavage with a second restriction enzyme or by strand separation using polyacrylamide gels as described elsewhere³⁸. Fragments were isolated and purified from these gels and subjected to chemical degradation according to Maxam and Gilbert²³. $\circ \rightarrow$ 3'-labelling, $\bullet \rightarrow$ 5'-labelling. *b*, Nucleotide and deduced amino acid sequences of the T3 δ -chain. The deduced amino acid sequence is shown below the nucleotide sequence. The N-terminal amino acid sequence derived from protein sequencing of the *in vivo* product is boxed and shown above the nucleotide sequence. The cysteine residues are circled and the dashed lines covered the stretches of hydrophobic amino acids. Possible sites for Asn-linked glycosylation are indicated ($\bullet$) and the polyadenylation signal (AATAAA) is underlined⁴¹.

cDNA, the 19-mer oligonucleotide mixture (probe 1) was used as primer for second strand synthesis (Fig. 1*b*). This approach generated a selected cDNA library of ~7,000 colonies in *Escherichia coli* MC1061. Subsequently, the library was screened with 32 P-labelled probes 1 and 2 and one cDNA clone hybridizing to both probes was isolated. The cDNA insert of this clone (pPGBC-1) was 0.62 kilobases (kb) long. In a subsequent experiment using the cDNA insert of pPGBC-1 as hybridization probe, one clone (pPGBC-8) that also hybridized with both oligonucleotide pools was isolated from a cDNA library comprising

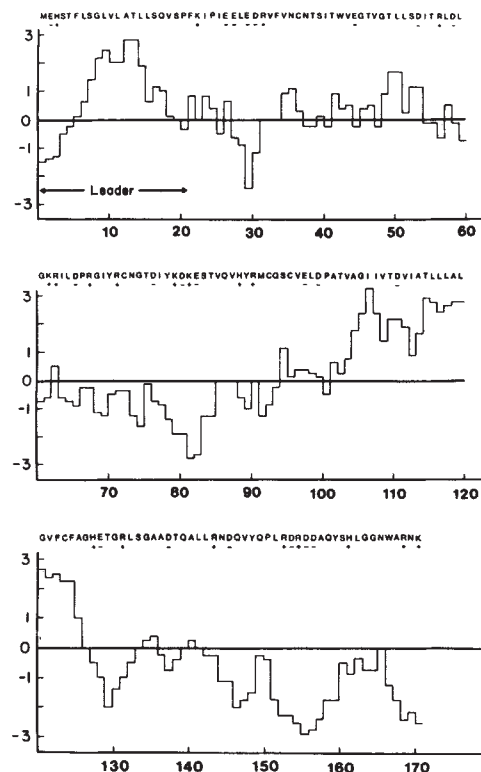


Fig. 3 Hydropathicity plot of the T3 δ -chain. The hydropathicity values of each amino acid have been determined using the weight system of Kyte and Doolittle⁴². The average hydropathicity value at residue *i* is calculated across 6 residues from *i*-3 through and including *i*+2. Positive values indicate hydrophobic regions and negative values represent hydrophilic segments. The one-letter amino acid nomenclature is used.

2×10^5 colonies made with messenger RNA from the human T-leukaemic cell line HPB-ALL. This cDNA clone, which has a 200-base pair (bp) insert, contained only the 5' end of the mRNA and has 111 nucleotides in common with the insert of pPGBC-1. We also used the insert of pPGBC-1 to screen a cDNA library which had been prepared from mRNA of a human T4⁺T8⁻ T-cell clone according to the method described by Okayama and Berg (refs 21, 22 and G. Nabel and J. Tseng, manuscript in preparation). From this amplified library of 30,000 primary colonies, one cDNA clone was isolated which contained an insert of 0.7 kb (pPGBC-9). To provide evidence that the cDNA clones were coding for the T3 δ -chain, nucleotide sequence analysis was conducted according to the methods designed by Maxam and Gilbert²³.

Nucleotide and protein sequences

Figure 2 shows the sequencing strategy and the nucleotide sequence of the 698 nucleotides of clones pPGBC-1, -8 and -9. The combined sequences of pPGBC-1 and -8 obtained from HPB-ALL cells and the sequence of pPGBC-9 obtained from a T4⁺T8⁻ T-cell clone are identical. A long open reading frame starts with an initiation coding ATG at position 97 and ends with a termination codon TGA at position 610. The 3'-untranslated region is 89 bp long, as indicated by the presence of an AATAAA sequence followed by a poly(A) tail 17 nucleotides downstream. The amino acid sequences from which the oligonucleotide probes were derived were found in the amino acid sequences predicted from the nucleotide sequence. Moreover, all 10 other amino acids known from the protein sequence data were found at the correct positions in the predicted amino acid sequence. Thus, the nucleotide sequence data provided conclusive evidence that the three cDNA clones encompass the coding information for the T3 δ -chain.

The predicted 171-amino acid sequence itself shows some interesting features. The 21-amino acid long N-terminal signal

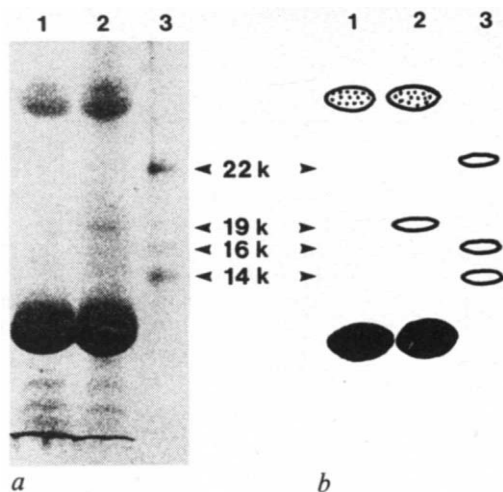


Fig. 4 SDS-polyacrylamide gel electrophoresis of the *in vitro* translation product of the T3 δ -chain mRNA. *a*, Lane 1, no RNA; lane 2, T3 δ -chain-selected mRNA; lane 3, Endo-F-treated anti-Leu-4 immunoprecipitate. *b*, Schematic representation of *a*. Numbers indicate the M_r values of the protein products discussed in the text.

Methods: 20 μ g of pPGBC-1, digested with *Pst*I, was spotted onto a nitrocellulose filter after denaturation. Poly(A)⁺ RNA was annealed to the bound T3 δ -chain cDNA in a buffer containing 70% formamide, 10 mM HEPES pH 6.8, 5 mM EDTA, 0.3 M NaCl and 0.2% SDS for 4 h at 47 °C. Following extensive washes with 1 $\times$ SSC, 0.5% SDS at 47 °C, the annealed RNA was released in a solution containing 90% formamide, 10 mM HEPES, 1 mM EDTA; 0.1% SDS and 25 μ g ml⁻¹ calf liver tRNA at 47 °C. T3 δ -chain-specific mRNA was isolated by ethanol precipitation in the presence of 0.2 M NH₄Ac, pH 5.5. The mRNA was translated in a rabbit reticulocyte system (BRL) in the presence of ³⁵S-L-methionine (Amersham). The products were electrophoresed in a 12.5% polyacrylamide SDS gel and compared with deglycosylated T3 proteins. For this preparation cells from the T-leukaemic cell line HPB-ALL were metabolically labelled with 1 mCi of ³⁵S-L-methionine and 1 mCi ³⁵S-L-cysteine (Amersham). The 20K proteins of the T3 complex were isolated by immunoprecipitation with the monoclonal antibody anti-Leu-4 as described previously⁸. The immunoprecipitate was subsequently treated with Endo-F as described elsewhere⁸. Following electrophoresis, the gel was treated with EN³Hance (NEN), dried and exposed to Kodak XAR-5 film at -70 °C.

peptide comprises a core of 19 hydrophobic amino acids which is flanked by two polar amino acids. Further analysis of the distribution of polar and nonpolar amino acids along the T3 δ -polypeptide chain (Fig. 3) reveals the presence of a hydrophobic segment (residues 80–106 in Fig. 2) which probably represents the transmembrane portion of the molecule. Although there is one aspartic acid residue (at position 90 in Fig. 2) in this stretch of hydrophobic amino acids, the negative charge of this amino acid does not influence the overall hydrophobic character of this stretch of amino acids (Fig. 3). The presence of charged amino acid residues in transmembrane segments has been observed in class I and class II antigens of the major histocompatibility complex (MHC) and T-cell receptor α - and β -chains^{4,6,7,24,25}. The unusual presence of a positively charged residue (Lys) in the T-cell receptor α - and β -chains suggests a possible salt bridge between one of these chains and the T3 δ -chain.

The first 79 N-terminal amino acids of the T3 δ -chain contain two potential sites for asparagine-linked oligosaccharides (residues 17 and 53, Fig. 2), in agreement with our previous Endo-H and Endo-F studies of the mature protein^{8,13}. The relative positions of the two oligosaccharide side chains and the transmembrane segment suggest that the 79 N-terminal amino acids are located on the extracellular side of the plasma membrane, and that residues 107–150 are intracellular. Interestingly, the intracellular sequence just C-terminal to the transmembrane

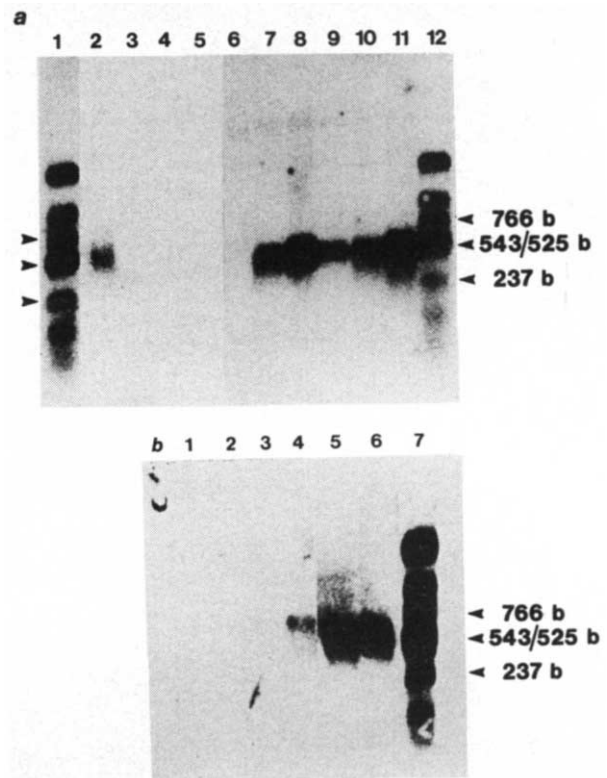
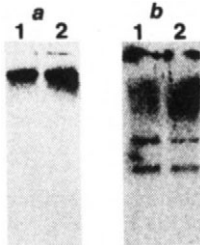


Fig. 5 Northern blot analysis of T-cell and non-T-cell RNA for the presence of T3 δ -chain specific mRNA. RNA from the following sources was used: *a*, Lanes 2, 8, human T-leukaemic cell line HPB-ALL; lane 3, HeLa cells; lane 4, human fetal brain; lane 5, human bone marrow; lane 6, human B-lymphoblastoid cell line JY; lane 7, human T-leukaemic cell line Molt-4; lane 9, T-leukaemic cells from a Sézary syndrome patient (T3⁺, T4⁺, T8⁻); lane 10, human thymus; lane 11, human T-leukaemic cell line JM-2. Lanes 1 and 12 represent a 5'-end-labelled *Hinf* digest of simian virus 40 DNA (SV40) DNA used as size markers. *b*, Lane 1, murine L-cells; lane 2, murine B-cell hybridoma A20; lane 3, murine T-T hybridoma (T-helper cell) B3C6; lane 4, murine T-T hybridoma (T-helper cell) B8C3; lane 5, human HPB-ALL cells; lane 6, human Molt-4 cells; lane 7, SV40 $\times$ *Hinf* size markers. Numbers on the right-hand side indicate the size (in bases) of some of the fragments of SV40 generated by *Hinf* digestion of 20 μ g of total RNA (*a*, lanes 2–5) or 4.5 μ g of poly(A)⁺ RNA (*a*, lanes 6–11, and *b*, lanes 1–6) were separated by electrophoresis in 1% agarose-formaldehyde gels³⁸ for 6 h at 5.5 V cm⁻¹. Following partial alkali hydrolysis, the RNA was transferred to nitrocellulose as described elsewhere³⁸. The blot was baked for 2 h at 80 °C, washed once in 3 $\times$ SSC, 0.2% SDS for 30 min at 65 °C and once in the same buffer for 30 min at 80 °C. The filters were prehybridized in a buffer containing 3 $\times$ SSC, 5 $\times$ Denhardt's solution, 0.5% SDS, 0.05% sodium pyrophosphate and 100 μ g ml⁻¹ of denatured salmon sperm DNA for 4 h at 65 °C. Hybridization was performed for 16 h at 65 °C in a buffer containing 3 $\times$ SSC, 1 $\times$ Denhardt's solution, 0.05% sodium pyrophosphate, 100 μ g ml⁻¹ denatured salmon sperm DNA and 5 $\times$ 10⁶ c.p.m. ml⁻¹ pPGBC-1 cDNA insert, ³²P-labelled by nick-translation. Following hybridization, the filters were washed three times at room temperature in 3 $\times$ SSC, 0.1% SDS and twice at 65 °C in the same buffer for 15 min each. The blots were exposed to Kodak XAR-5 film at -70 °C using an intensifying screen.

region does not contain the stretch of positively charged amino acids frequently observed in other membrane proteins²⁵.

Comparisons between the nucleotide and protein sequences of the T3 δ -chain and the T-cell receptor α - and β -chains using the NUCALN and PRTALN programs show no strong homologies and no significant homologies between the T3 δ -chain and immunoglobulin polypeptide chains or rat and murine Thy-1. The latter glycoprotein has often been thought of as the murine T3 equivalent^{27,28}. Further searches using the Wilbur-Lipman search program for homologous protein sequences in

Fig. 6 Genomic representation of T3 δ -chain DNA from HPB-ALL cells or human thymus was used to analyse T3 δ -chain-specific sequences. Lanes 1, HPB-ALL DNA; lanes 2, thymus DNA. 10 μ g of cellular DNA was digested with *Eco*RI (a) or *Bam*HI (b). DNA fragments were separated in a 1% agarose slab gel and blotted onto nitrocellulose after denaturation of the DNA in the gel^{43,44}. T3 δ -chain-specific sequences were detected by hybridization with a ³²P-labelled T3 δ -chain cDNA insert of pPGBC-1.



the Dayhoff database were unsuccessful²⁹. The T3 δ -chain, unlike the α - and β -chains of the T-cell receptor, therefore seem not to belong to the immunoglobulin/MHC/T-cell-receptor multi-gene family.

Protein processing

The M_r of the protein backbone of the T3 δ -chain was estimated from the predicted amino acid sequence at 16,719, apparently larger than the 14K measured for the deglycosylated membrane bound T3 δ -chain. We therefore determined the size of the protein product of the T3 δ -chain mRNA following *in vitro* translation. Poly(A)⁺ mRNA from HPB-ALL cells was hybridized to clone pPGBC-1 immobilized onto a nitrocellulose filter. After removal of the unattached mRNA by several washes, the annealed mRNA was released and translated *in vitro* in a rabbit reticulocyte system. Figure 4 (lanes 2) shows that a 19K protein was synthesized from this mRNA and migrated considerably more slowly than the deglycosylated T3 δ -chains—the 14K and 16K products (lanes 3)—because the *in vitro* translation product contained the signal peptide (2.2K) which is not removed by proteolysis in the reticulocyte translation system. This demonstrated that the T3 δ -polypeptide chain indeed has a M_r of 16K. Because we consistently find a protein backbone of 14K on deglycosylation of the membrane-bound T3 δ -chain, a proteolytic step must have occurred to convert the 16K into the 14K backbone. As we have determined previously that the 16K product had an N-terminal sequence identical to the 14K species, it seems that a C-terminal fragment of the T3 δ -chain is removed by proteolysis.

Expression of T3 δ -chain mRNA

Monoclonal antibodies directed at the T3 complex (OKT3, Leu-4, UCHL1) have shown that it is present on all human peripheral blood thymocytes, medullar thymocytes and to a lesser extent on cortical thymocytes³⁰⁻³³. To examine expression of the T3 δ -chain mRNA, several haematopoietic and other cells were analysed by Northern blot analysis (Fig. 5). T3 δ -chain mRNA was detected in thymocytes, peripheral blood lymphocytes and T-leukaemic cells, but not in the B-lymphoblastoid cell line JY, bone marrow cells, HeLa cells or fetal brain tissue (Fig. 5a). This demonstrated that the T3 δ -chain mRNA is present in cells of T lineage only.

Studies with monoclonal antibodies directed at murine T lymphocytes have not resulted in the detection of a murine T3 equivalent. This is somewhat surprising in view of the strong structural similarity between the human and murine T-cell receptor α - and β -chains¹⁻⁶. Northern blot analysis using the insert of the cDNA clone pPGBC-1 as hybridization probe revealed

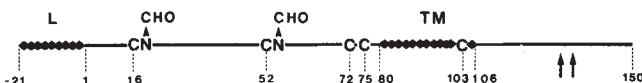


Fig. 7 A model for the T3 δ -chain. General features of the organization of the T3 δ -chain. CHO are the N-linked glycosylation sites. The arrows indicate the area of proteolytic processing. Stretches of hydrophobic amino acids are represented by the series of diamonds. L represents the leader sequence, TM the transmembrane region and C and N, cysteine and asparagine residues respectively. The numbers refer to the relative positions of the methionine initiator codon, the first amino acid of the mature protein, the termination codon, the position of the cysteine residues and the transmembrane region.

a 0.7-kb mRNA in the T-T hybridoma lines (T helper) B8C3 and B3C6 (Fig. 5b). In contrast, no obvious hybridization was observed with RNA from B-cell hybridoma A20 or from L-cells.

Taken together, the analysis of human and murine mRNAs shows that expression of the T3 δ -chain is restricted to T lymphocytes. Furthermore, the existence of a T3 δ -chain mRNA isolated from murine lymphocytes provides the first evidence that a murine T3 δ -chain equivalent exists.

Genomic representation of T3 δ -chain

Genomic DNA isolated from HPB-ALL cells and thymocytes was analysed by Southern blot analysis after digestion with *Eco*RI and *Bam*HI, respectively. The T3 δ -chain is located in both HPB-ALL cells and thymocytes on a single 13-kb *Eco*RI fragment (Fig. 6a). Digestion with *Bam*HI, which has a cleavage site in the deduced cDNA sequence at position 396, produces only two fragments of 3.3 and 2.2 kb (Fig. 6b). These findings indicate that the T3 δ -chain is represented as a single-copy gene in the genome.

Discussion

Several conclusions can be drawn from the amino acid sequence derived from cDNAs encoding the T3 δ -chain. The transmembrane segment consists of a stretch of 27 predominantly hydrophobic amino acids (residues 80–106 in Fig. 2) which is interrupted by an aspartic acid (residue 90 in Fig. 2). Asp 90 could form a salt bridge with the Lys residue in the transmembrane segment of the T3/T-cell receptor α - or β -chain^{4,6,7}. This type of bond would be pronounced due to the low dielectric constant of the hydrophobic environment. Unlike several other transmembrane segments, the T3 δ -chain hydrophobic domain does not contain a high level of α -helix-forming residues. Furthermore, this intracellular domain is not immediately followed by a string of positively charged amino acids, as in most transmembrane proteins²⁶. Perhaps association with the other transmembrane chains of the T3/T-cell receptor complex compensates for the need for such a positively charged area in the molecule. It is unlikely that the T3 δ -chain has a hairpin configuration, because protease K experiments with 'inside-out' microsomal vesicles showed that all components present in anti-T3 immunoprecipitates span the plasma membrane¹⁴. The T3- δ -chain can therefore be divided into two functional domains: an extracellular one of 79 amino acids, with two N-linked oligosaccharides, and a cytoplasmic domain of 44 amino acids (Fig. 7). This cytoplasmic domain is larger in relation to the extracellular compartment than are the T3/T-cell receptor α - and β -chains, perhaps reflecting the signal transferring function of the invariable T3 chains as opposed to the ligand binding function of the clonotypic T-cell receptor heterodimer.

The primary translation product of the T3 δ -chain has a M_r of 19K, including a 2.2K signal peptide. This observation, combined with our previous finding that on deglycosylation a 16K and a 14K product are formed, strongly suggests a proteolytic processing step. Indeed, pulse-chase experiments have shown that a 23K intermediate may be converted into one of the 20K T3 bands. This 23K species could be converted to a 16K protein backbone with Endo-H whereas the 20K species could be converted into the 14K protein⁸. In contrast, mature 20K T3; isolated from a membrane fraction was always converted into a 14K protein after treatment with Endo-F. As the 14K and 16K T3 fragments have the same N-terminal sequences, the proteolytic processing must have occurred in the C-terminal cytoplasmic portion of the T3 δ -chain (Fig. 7). In view of the role of T3 in T-cell activation, it is interesting to speculate that this proteolytic processing may be involved in the activation of T lymphocytes following the binding of antigen to the T-cell receptor.

The distribution of the half-cysteine residues is somewhat unusual in that two are directly N-terminal to each of the asparagine-linked oligosaccharides. Another cysteine lies in the transmembrane region, a characteristic of several of the MHC class II antigens²⁴. Protein fragmentation studies (J.B. and C.T., unpublished) suggest that intra-chain sulphhydryl bridges may

exist between some of the five half-cysteines, although it is not known how these bridges are arranged within the molecule. There is no evidence for their involvement in inter-chain disulphide bridges with the other chains of the T3 complex.

The gene for the T3 δ -chain seems to be a single-copy element expressed in all T lymphocytes. Furthermore, comparisons with other known protein sequences indicate that the T3 δ -chain is a unique polypeptide. Several groups have argued that T3 has an analogous role to Thy-1 in the murine T lymphocyte. Indeed, monoclonal and conventional anti-Thy-1 antibodies are mitogenic for murine T lymphocytes^{27,28}, as are anti-T3 reagents. However, the data presented here demonstrate that no sequence homology exists between the T3 δ -chain and Thy-1. In addition, whereas the Thy-1 antigen is expressed on neuronal cells, the T3 δ - and the ϵ -chains are not¹⁶. As the T3 ϵ -chain is not glycosylated and is probably a protein with multiple membrane segments, the only possible Thy-1-like member of the T3 complex is the 25K T3 γ -chain.

Our data demonstrate the existence of a murine equivalent of the human T3 δ -chain. The fact that this polypeptide chain has never been detected on the cell surface with murine antibodies specific for murine T lymphocytes may be due to its lack of genetic polymorphism. Indeed, comparison of human T3

δ -chains from different individuals by isoelectric focusing has not revealed any polymorphism in man¹².

The structural studies described here provide a basis from which to study the biosynthesis, surface expression and function of the T3 complex in a variety of different lymphocyte clones, and during T-cell differentiation. The extent of association between the T3 δ -chain and the clonotypic heterodimer is obviously determined by the properties of all five components of the T3/T-cell receptor, and this association may in fact govern the functional response following the binding of antigens to its receptor. In such a model, the T3 δ -chain may be involved in transducing a signal to the intracellular compartment.

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Novel immunoglobulin heavy chains are produced from DJ_H gene segment rearrangements in lymphoid cells

Michael G. Reth & Frederick W. Alt

Department of Biochemistry and Institute for Cancer Research, Columbia University, College of Physicians and Surgeons, New York 10032, USA

It has been found that most immunoglobulin heavy(H)-chain gene diversity (D) segments carry their own 5' transcriptional promoter element. Transcription of rearrangements which use heavy-chain D and joining (J) segments in B-lymphoid cells ultimately leads to the production of a $D\mu$ messenger RNA which contains the DJ_H segments linked to the μ heavy-chain constant region. If the D is joined to the J_H segment in the appropriate translational reading frame, this $D\mu$ mRNA is translated to yield a short $D\mu$ protein with a variable DJ_H N-terminus.

THE variable region of a classical immunoglobulin heavy-chain gene is assembled from three germ-line components: the variable (V_H) segment, the diversity (D) segment and the joining (J_H) segment¹⁻³. These segments lie upstream of the constant (C_μ) region. The D segment has an important role in this somatic gene assembly process by mediating the joining of V_H and J_H

elements. This joining process is ordered: a D element combines first with a J_H element to form a DJ_H complex to which a V_H element is subsequently appended⁴. In the expressed V_H domain, the D sequence encodes part of the third complementarity-determining region and is thus important in generating antibody diversity⁵. Twelve D elements, 1-80 kilobases (kb) upstream

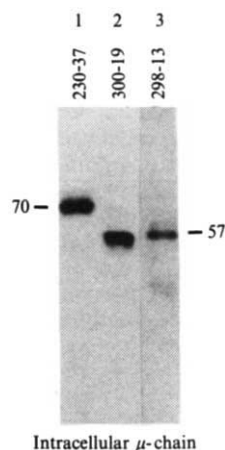


Fig. 1 Expression of truncated heavy-chain gene products in A-MuLV transformants. Approximately 10^7 cells were lysed in phospholysis buffer³⁴, the lysates reduced in the presence of mercaptoethanol, fractionated by electrophoresis through 10% polyacrylamide gels³⁵ and electrotransferred to a nitrocellulose filter for 4 h at 150 V in a Transblotter (Biorad). Nonspecific binding sites on the filter were saturated by a 5-h incubation in a 10% casein/phosphate-buffered saline (PBS) solution and the bound μ chains detected by a sandwich technique using a goat anti-IgM antiserum (a gift of A. Radbruch) and 125 I-labelled monoclonal IgM antibody B1-8 (ref. 36).

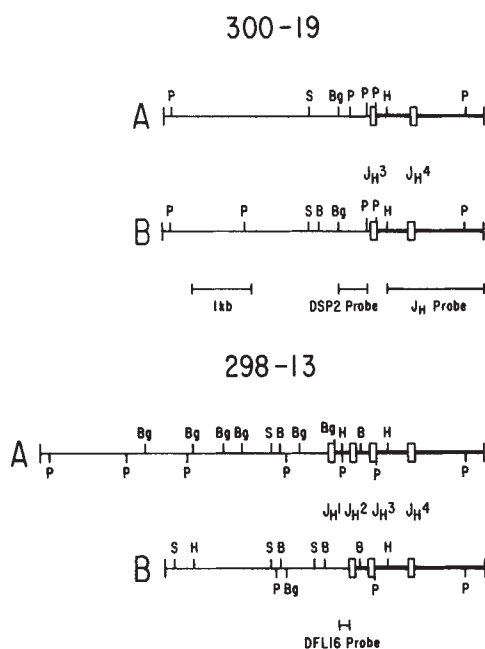


Fig. 2 Restriction maps of cloned DJ_H alleles of 300-19 and 298-13. The two DJ_H rearrangements in each of these lines were arbitrarily designated as A or B. Individual rearrangements were isolated by cloning complete *EcoRI*-digested genomic DNA from the appropriate lines into Charon 16a and screening the resulting libraries for recombinants which hybridized to a J_H probe as described previously⁹. Inserts from the appropriate recombinant phage were subcloned into PUC-9 and partial restriction maps constructed by single, double or triple digestions of the plasmid DNA with the indicated restriction enzymes (B, *Bam*HI; Bg, *Bgl*II; H, *Hind*III; P, *Pst*I; S, *Sac*I). The J_H part of the DJ_H complex is indicated by a darker line. The location of the J_H , DSP2 and DFL16 probes are indicated below the restriction maps; the DSP2 probe is a 500 bp *Bgl*II-*Bst*EII fragment from the DSP2 J_H rearrangement of P4-12 (see Table 1) which had a *Bst*EII site at the DJ_H joint. The DFL15 probe is a 100 bp *Ava*II-*Sau*3A fragment, spanning the region -120 to -10 in Fig. 3a.

from the J_H locus, have been described in the mouse genome⁶. These elements comprise three families (5' to 3'), DFL16 (two members), DSP2 (nine members) and DQ52 (one member)⁷.

Transformation of bone marrow or fetal calf liver cells with Abelson murine leukaemia virus (A-MuLV) generates permanent lymphoid cell lines. Most transformants represent the pre-B stage of B-lymphocyte differentiation and provide a model system for the study of the early stages of immunoglobulin gene rearrangement and expression⁸. Some A-MuLV-generated lines produce an unusually small μ heavy-chain messenger RNA and sometimes a small μ protein⁹. We report here that the short μ mRNA sequences arise from the transcription of DJ_H rearrangements and the short μ proteins from the translation of the resulting $DJ_H C_\mu$ -containing mRNAs ($D\mu$ mRNA). Because most of the D segments have the potential for expression after juxtaposition to a J_H segment, we suggest that they may have a function independent of their association with a V_H gene segment.

A short μ -chain in A-MuLV transformants

We studied two lines which produce short μ -chains, 300-19 and 298-13 (ref. 10), derived from the bone marrow of NIH/Swiss and BALB/c mice, respectively. The size of these μ -chains was determined by fractionating cell lysates from each line by electrophoresis through SDS-polyacrylamide gels, transferring to nitrocellulose paper¹⁰ and detecting immunoglobulin chains by a sandwich technique using anti-IgM antisera and 125 I-labelled monoclonal IgM antibody. We also analysed lysates from a control transformant (230-37) which produces normal-sized μ -chains of molecular weight $\sim 70,000$ (70K) (Fig. 1, lane 1). Both the 300-19 and 298-13 lines clearly contained abnormally small μ proteins of approximately 57K (Fig. 1, lanes 2, 3). Furthermore, we confirmed that instead of the normal 2.4 and 2.7 kb μ mRNAs which encode, respectively, the secreted and membrane-bound forms of the μ protein^{11,12}, the 300-19 and 298-13 lines contain truncated C_μ -specific RNAs of 2.0 and 2.3 kb; these species contain 3' ends specific to the membrane and secreted forms of the protein, respectively⁹. We suggest that the truncated μ -chains are encoded by correspondingly truncated μ mRNA species.

Production of the short μ -chain

Rearrangements of the J_H alleles in the 300-19 and 298-13 lines were detected and characterized by genomic DNA blotting assays using D - and J_H -specific probes as described previously⁴. These analyses indicated that each line had DJ_H rearrangements at both J_H alleles (data not shown) and thus represented a very early stage of B-cell differentiation⁴. To elucidate these rearrangements further, we cloned *EcoRI*-digested genomic DNA from the two lines into λ phage Charon 16A and isolated recombinants which hybridized to a J_H -specific probe. We show the restriction maps of the cloned DJ_H alleles (denoted A and B) of each line (Fig. 2) and the nucleotide sequence around the DJ_H joints (Fig. 3a). These data indicate that both DJ_H rearrangements of the 300-19 line involved a J_H3 segment and similar yet distinct DSP2 elements, whereas the DJ_H rearrangements on the A and B allele of the 298-13 line involved joining of DSP2 to J_H1 and DFL16.1 to J_H2 , respectively. All four of these D elements carried the conserved recognition heptamer and nonamer sequences separated by the characteristic 12 base pair (bp) spacer². The three DSP2 segments have a very similar 5' flanking sequence which differed from DFL16 in over 30% of the nucleotide positions. Each DJ_H joint occurred in different positions of the D or J_H germ-line sequence, indicating the flexibility of the joining process^{13,14}. For example, on allele A of 300-19 (Fig. 3a), the rearrangement event resulted in the deletion of most of the DSP2 coding sequence and a large part of the J_H sequence. This allele also had a 12-nucleotide sequence between the D and J_H segments which was not present in the germ-line sequence of either element; such 'N-region' sequences (cross-hatching in Fig. 3) are thought to be generated *de novo*

Table 1 Expression of $D\mu$ RNA and $D\mu$ chain in lymphoid cell lines and tissues

Cell line	Rearrangements		DSP RNA	DFL RNA	$D\mu$ protein
	Allele A	Allele B			
Bone marrow					
300-19	DSPJ _H 3	DSPJ _H 3	+	-	+
300-19P3	—	DSPJ _H 3	+	-	+
300-19P18-7-7	DSPJ _H 3	—	+	-	-
300-19P8	VDJ _H 3	VDJ _H 3	-	-	-
298-13	DSPJ _H 1	DFLJ _H 2	+	+	+
298-13-9	VDJ _H 3	DFLJ _H 2	-	+	+
P4-12	DSPJ _H 4*	DFLJ _H 4*	+	+	-
18-48	DFLJ _H	—	-	+	-
1-8	VDJ _H	DFLJ _H	-	ND	-
3-1	VDJ _H	VDJ _H	-	ND	-
298-8	VDJ _H	VDJ _H	-	ND	-
230-37	VDJ _H	VDJ _H	-	ND	-
Fetal liver					
22D6.10	DFLJ _H 4*	DSPJ _H 3*	+	ND	-
22D6.C11	DFLJ _H 4	DSPJ _H 4	+	ND	+
22D7	DSPJ _H	DFLJ _H	+	ND	-
38B11	DFLJ _H	DSPJ _H	+	ND	-
38B9	DFLJ _H *	DSPJ _H	+	ND	-
40E1	DSPJ _H	DSPJ _H	+	ND	-
40E4	DSPJ _H *	DSPJ _H *	+	ND	-
40E3	DFLJ _H	DFLJ _H	-	ND	-
41B1	DFLJ _H	DFLJ _H	-	ND	-
38B7	VDJ _H	VDJ _H	-	ND	-
Myeloma					
J606	DSPJ _H	VDJ _H	+	ND	ND
H2020	VDJ _H	VDJ _H	-	ND	ND
MPC11	VDJ _H	VDJ _H	-	ND	ND
M315	VDJ _H	VDJ _H	-	ND	ND
T-cell lines					
TB1	DSPJ _H	—	+	ND	-
TB2	DJ _H	DJ _H	+	ND	-
TB3	DJ _H	DJ _H	+	ND	ND
Tissue					
15-Day fetal liver			+	ND	ND
21-Day fetal liver			+	ND	ND
Adult spleen			+	ND	ND
21-Day thymus			+	+	ND
Adult thymus			+	+	ND

The rearrangement status of the J_H alleles (A and B) in A-MuLV transformants and myeloma lines were analysed by Southern blotting, using D - and J_H -specific probes as described previously⁴. The presence of $D\mu$ RNA in various cell lines and tissues was detected by a slot blot procedure. Briefly, 5 μ g of total RNA or 0.5 μ g of poly(A)-containing RNA was denatured in 6% formaldehyde, 50% formamide and 1×SSC at 68 °C for 10 min, diluted 4× with 20×SSC and then blotted onto nitrocellulose filters using a slot-blotting apparatus (Schleicher & Schull, minifold 2). The filters were then baked, incubated with DSP2- or DFL16-specific probes (see Fig. 2 for description of probes) and washed as described previously⁹. The presence of the $D\mu$ chain was analysed by Western blotting as described in Fig. 1 legend. The presence or absence of $D\mu$ RNA and $D\mu$ chain is indicated by + and - in the appropriate column. Deletion of one of the J_H alleles in certain lines was defined by the presence of only one J_H -positive band in a Southern blot of genomic DNA cut by a panel of restriction enzymes and is indicated by a - in the rearrangement column. ND, not done.

* These J_H alleles were cloned, sequenced and proven to have an out-of-phase DJ_H joint as described in the text (ref. 33 and M.G.R. and F.W.A., in preparation).

during the D to J_H joining process¹⁵. N regions were also present in two of the other DJ_H rearrangements and contributed significantly to the variability of the DJ_H complex (Fig. 3a). These sequencing analyses demonstrated unequivocally that both J_H rearrangements of 300-19 and 298-13 were DJ_H joints and suggested that the short μ transcripts and proteins in these lines were derived from a DJ_H rearrangement. We will now refer to these products as $D\mu$ mRNA and $D\mu$ protein.

We next determined whether the $D\mu$ chain was encoded by one or both DJ_H alleles in 300-19 and 298-13. This analysis was possible because both lines, like fetal liver-derived A-MuLV transformants with two DJ_H rearrangements⁴, undergo 'secondary' rearrangements of their DJ_H complexes during propagation in culture (M.G.R. and F.W.A., in preparation). These secondary rearrangement events include appendage of V_H segments to the DJ_H complexes with the accompanying deletion of all sequence lying between the joined V_H and DJ_H segments⁴ and non- V_H -mediated deletion events which result in the complete loss of one of the DJ_H alleles as well as the flanking D and J_H segments. The nature of each J_H -specific secondary

rearrangement in a collection of 300-19 and 298-13 subclones was determined by the Southern blotting assay described previously⁴ and compared with μ -chain expression in the respective lines (Fig. 4). Digestion of total cellular DNA simultaneously with *Bam*HI and *Eco*RI liberates the DJ_H A and B alleles of the 300-19 parent line as distinguishable J_H -specific fragments of 5.5 and 2.8 kb (Fig. 4A, lanes 1; see Fig. 2 for description of J_H probe and maps of rearrangements). Both fragments are clearly different from the 2.1 kb unrearranged, J_H -positive fragment observed in *Bam*HI-*Eco*RI-digested liver DNA (Fig. 4A, lanes labelled L).

Analysis of two subclones of the 300-19 line which, because of non- V_H -mediated deletion events, contained only the DJ_H A allele (P18-7-7) or the DJ_H B allele (P3), demonstrated that only the line containing the latter allele produced the $D\mu$ chain (Fig. 4A, lanes 2, 3; compare a and b). This result indicates that the $D\mu$ chain in 300-19 is derived from the DJ_H B but not the DJ_H A rearrangement (although both DJ_H alleles transcribe a $D\mu$ mRNA). A study of 300-19 daughter cells which underwent V_H to DJ_H rearrangement during propagation in culture added

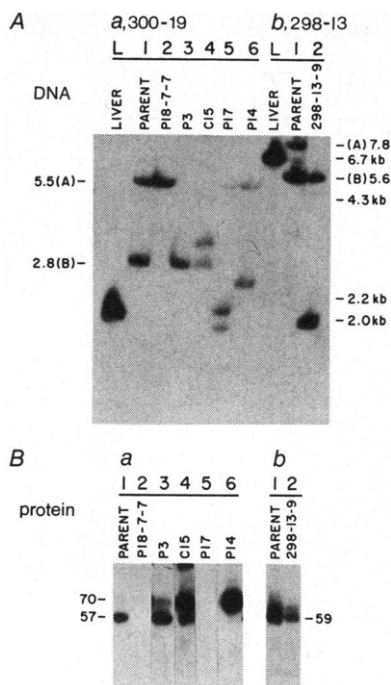


Fig. 4 J_H rearrangements and intracellular μ chains in subclones of 300-19 and 298-13 lines. Subclones of the 300-19 and 298-13 lines were isolated by limiting dilution as described previously⁸. Deletion of or V_H appendage to the pre-existing DJ_H rearrangements of these lines was determined by the genomic blotting assays described previously⁴. **A**, DNA: Approximately 10 μ g of genomic DNA was digested either with a combination of the restriction enzymes *Bam*HI and *Eco*RI (*a*) or with *Eco*RI alone (*b*), electrophoresed through 1% agarose, blotted onto nitrocellulose and assayed for hybridization to a ³²P-labelled J_H probe (see Fig. 2). The bands corresponding to the two parental DJ_H alleles in each line are indicated as A and B, respectively. Other lanes contained DNA from individual subclones with either a deletion or a V_H to DJ_H rearrangement of the parental DJ_H alleles as described in the text. **B**, Protein: The cell lysate of each line analysed was fractionated by polyacrylamide gel electrophoresis, transferred to nitrocellulose, and bound μ heavy chains detected by ¹²⁵I-labelled antibodies as described in Fig. 1 legend.

adenine residue situated 89 nucleotides upstream of the D heptamer (position -89, asterisk in Fig. 3a). The promoter-like sequences TAAAAAG (300-19, both alleles) and TAAAAAT (298-13, allele A) were found 45 nucleotides upstream of the $DSP2$ transcriptional initiation site. Although the 300-19 $DSP2$ sequence contains two other TATA box-like elements (underlined in Fig. 3a), we regard the above sequences as the most likely $DSP2$ promoter, because we also found similar elements (GAAAAAG and TAAAAAG) in the 5' sequence of the FL16- and Q52-type D segments. Both of the latter D elements are transcriptionally active in cells which carry the respective DJ_H rearrangement, such as DFL16 (Table 1) and DQ52¹⁶. The 2.3 and 2.0 kb μ -chain-specific RNA sequences in 300-19 and 298-13 contain the four C_μ coding exons plus either a μ_m or μ_s 3' terminus (where m and s represent membrane-bound and secreted forms), respectively; these mRNA sizes are consistent with RNA transcripts initiated at the indicated D promoter elements and subsequently spliced to yield a 5' DJ_HC_μ 3' RNA sequence ($D\mu$ mRNA). In support of this conclusion, we find that the 2.3 and 2.0 kb C_μ -specific RNA sequences in these lines hybridize to probes specific for the putative D portion of the transcript (data not shown).

Analysis of the 5' $DSP2$ sequence revealed an ATG codon 26 nucleotides downstream of the transcriptional initiation site (position -63 in Fig. 3a) which is the beginning of an open reading frame throughout the following D sequence. Further-

more, the $DSP2J_H$ joint of allele B in 300-19 as well as the $DSP2J_H$ rearrangement of 298-13 (each of which were linked to the short μ -chain product, as described above) placed this ATG in phase with the known translational reading frame of the J_H coding sequence to which it was appended. The $DSP2J_H$ joint of allele A of 300-19, on the other hand (which does not encode a $D\mu$ chain), places the -63 ATG of the $DSP2$ element in a different translational reading frame from that of the linked J_H coding sequence (Fig. 3a). Additional studies with several other subclones and independent cell lines which contained $DSP2$ rearrangements indicated that only those which had the -63 ATG sequence in the same translational reading frame as the linked J_H sequence produced the $D\mu$ protein, although all produced $DSP2\mu$ RNA sequences (Table 1).

An ATG codon is not found at the -63 position in the DFL16 sequence; an ATG codon situation 48 nucleotides farther upstream (at position -109), however, divides the following D sequence into the same translational reading frame as does the $DSP2$ ATG at -63. In the 298-13 productive DFL16 J_H rearrangement, this ATG is in the same translational reading frame as the J_H coding region. In several other lines which had DFL16 J_H rearrangements but which did not produce $D\mu$ chains, the DFL16 ATG was out of phase with the J_H coding sequence (Table 1). We conclude that the $DSP2$ ATG at -63 and the DFL16 ATG at -109 are the translation initiation sites of the $DSP2$ - and DFL16-type $D\mu$ chain, respectively. These ATG sequences represent the first (most 5'-proximal) translation initiation codons on the respective $D\mu$ mRNAs and both ATG codons are flanked by 5' sequences (a conserved A or G residue three nucleotides upstream) that are important for efficient utilization¹⁷, which supports our conclusions.

Expression of $D\mu$ RNA and $D\mu$ protein

We used probes for SP2- and FL16-type D elements derived from the body of D and the immediate 5' flanking region to detect $D\mu$ mRNA in various B-lymphoid cell lines (Fig. 2). We find a strict correlation between the presence of the $D\mu$ mRNA and a DJ_H rearrangement (Table 1). $DSP2\mu$ mRNA is found in subclones of 300-19 (P3 and P18-7-7) with a deletion of either the A or the B allele, which proves that each 300-19 DJ_H allele is transcriptionally active. In 300-19 subclone P8, appendage of V_H segments to the DJ_H rearrangements of both alleles resulted in deletion of all D promoter sequences and $D\mu$ mRNA transcripts are no longer produced (Table 1). The 298-13 parent line produces both $DSP2$ - and DFL16-type μ chains, thus it contains both $DSP2$ - and DFL16-type $D\mu$ mRNA. A subclone (298-13-9) with a non-productive rearrangement of the $DSP2$ allele lost the $DSP2$ transcript but retained the FL16-type mRNA. Analysis of many different lines consistently showed that transformants with V_HDJ_H rearrangements at both J_H alleles (mostly of adult marrow origin) did not produce $D\mu$ mRNA sequences, whereas transformants with one or more DJ_H rearrangements (mostly of fetal liver origin) always produced $D\mu$ mRNA (Table 1). Many of the lines analysed contained $D\mu$ mRNA but did not produce the $D\mu$ protein. Both J_H alleles from three such lines were cloned, sequenced and proven to have an out-of-phase (non-productive) D to J_H joint with respect to putative 5' D translation initiation codons (Table 1).

We included four myeloma cells (representing terminally differentiated B cells) in the RNA analysis and found that the one line (J606) containing a $DSP2J_H$ rearrangement⁴ also produced $D\mu$ mRNA (Table 1). T-lymphoid cells frequently contain DJ_H rearrangements¹⁸⁻²⁰; we have found $D\mu$ mRNA in three T-cell TB lines²¹ that have these rearrangements and in RNA extracted from normal lymphoid tissues such as adult spleen, fetal liver and newborn thymus (Table 1). The expression of $D\mu$ mRNA in spleen and fetal liver is consistent with our data derived from the various B-cell lines. The detection of $D\mu$ message in the newborn thymus, where contamination by B-lymphoid cells should be negligible⁹, agrees with the previous finding of significant quantities of 2.3 and 2.0 kb C_μ -containing

RNA sequences in this tissue^{9,22,23}, suggesting that some normal T-lymphoid cells produce $D\mu$ mRNA sequences.

Function of $D\mu$ chains

We have demonstrated that most D elements have a 5' transcriptional promoter. Like the V_H promoter, the D promoter is presumably activated if a DJ_H rearrangement places it in close proximity to the IgH enhancer element^{24,25,40,41}. When the DJ_H joint places the 5' D translation initiation codon in the same translational reading frame as the J_H coding sequence, the $D\mu$ mRNA encodes a short μ -protein. The definition of D segments as mini-genes²⁶ raises the question of what function the short $D\mu$ chain may have. The first 18 amino acids of the DSP2-derived μ -protein sequence have a striking similarity to known leader peptides²⁷, containing a stretch of 10 hydrophobic residues flanked by charged amino acids (Fig. 3b). We conclude that the DSP2 μ chain is inserted into the endoplasmic reticulum, supported by the observed glycosylation of this chain⁹. Although the $D\mu$ chain lacks a classical variable-region domain, it has a variable DJ_H peptide followed by the four μ -chain constant region domains and a membrane or secreted C-terminus. The DFL16-derived $D\mu$ protein differs from the DSP2 μ chain only in the N-terminus, which is 15 amino acids longer and starts with a different leader-like sequence (Fig. 3b). The structural consequence of this difference is that the hypervariable region of the DSP2 μ chain is close to the N-terminus whereas in the DFL16 μ chain it is flanked on the N-terminus by conserved D sequences. RNA from the 300-19 line (which only produces DSP2 μ chains) programmes synthesis of two distinct μ chains of 52K and 55K molecular weight whereas RNA from the 298-13 line (which produces both DSP2 μ and DFL16 μ chains) programmes four μ -chain translation products of 52K, 55K, 54K and 57K⁹. The translation products of the normal membrane and secreted forms of μ -chain mRNA are 67K and 64K, respectively^{11,12}; the 52K and 55K μ chains presumably represent the products of the membrane and secreted forms of DSP2 μ mRNA while the 54K and 57K chains represent those of the corresponding forms of DFL16 μ mRNA. The expression of the $D\mu$ protein is not subject to allelic exclusion in the 298-13 line, which supports the proposal that V_H to DJ_H joining is the allelically excluded step in V_H gene assembly^{4,9}.

$D\mu$ proteins can be expressed in conjunction with a C-terminus specific for the membrane form of the constant region, suggesting that they may be part of a novel, variable receptor system; however, we have not yet found the protein expressed on the surface of A-MuLV transformants. The normal-sized μ chain is found on 300-19 surfaces only with an immunoglobulin light chain (M.G.R. and F.W.A., in preparation), which suggests that a second chain may be necessary for surface expression. The detection of $D\mu$ transcripts in neonatal thymus (Table 1) may explain the presence of immunoglobulin-like chains on the surface of T cells^{28,29}. DJ_H rearrangements in T cells are generally thought to have no function³; our suggestion that they may encode a $D\mu$ protein warrants a re-evaluation of this idea. Surface $D\mu$ -chain expression could also contribute to the observed idiotypic cross-reaction between T and B cells³⁰.

Sequence variations between D elements expressed in the mouse strains used in our study maintained the positive charge of the putative leader peptide and did not impair $D\mu$ -chain expression (Fig. 3b). A sequence comparison of homologous murine and human DQ52 segments shows only a few stretches of homology³¹, one of which contains the D promoter-like sequences. Another human D element ($D36$)³¹ contains 5' promoter and ATG sequences in positions similar to the mouse DSP2 segment, although its expression is probably prevented by an in-phase translation stop codon. However, a short μ chain similar to the murine $D\mu$ chain has been found in human pre-B-cell lines derived from patients with X-linked agammaglobulinaemia, possibly as a result of incomplete V_H to DJ_H joining⁴². This evolutionary conservation of $D\mu$ -chain expression also implies a functional significance for this chain. D segments of the β chain of the T-cell receptor are also transcribed after the formation of a DJ_β rearrangement; the 5' flanking region of these segments also has potential translation initiation sites³². Thus the T-cell DJ_β rearrangements similarly may lead to the production of DJ_β polypeptide chains.

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Regulation of actin polymerization by non-polymerizable actin-like proteins

Hiroshi Maruta, Wiebke Knoerzer, Horst Hinssen* & Gerhard Isenberg

Max-Planck-Institute for Psychiatry, D-8033 Martinsried/Muenchen, FRG

*Institute for Molecular Biology, Austrian Academy of Sciences, A-5020 Salzburg, Austria

Three functionally distinct actin-capping proteins from the slime mould Physarum are structurally closely related to actin itself. In Physarum, actin polymerization is regulated by a set of non-polymerizable actin-like proteins. It remains to be established whether these proteins and actin are each encoded by separate genes.

PHYSARUM contains at least four distinct actin-binding proteins of approximately 42,000 molecular weight (MW)—actin, Cap42(b), Cap42(a) and fragmin¹⁻⁴. Only actin, which is present abundantly, forms filaments by self-assembly. The other three proteins do not form filaments but instead are able to block elongation of actin filaments by capping the fast-growing end of the filaments in a Ca^{2+} -dependent process²⁻⁵. Fragmin has an additional F-actin severing activity which leads to a rapid fragmentation of actin filaments when Ca^{2+} is present^{2,6-8}. Cap42(b) is phosphorylated by a specific kinase from *Physarum*; this phosphorylation changes its F-actin capping activity. Cap42(b) requires Ca^{2+} for its F-actin capping activity only when it is fully phosphorylated (when it is dephosphorylated, its F-actin capping activity is Ca^{2+} -independent)¹⁻⁴. The Cap42(b) kinase is highly specific for Cap42(b) and never phosphorylates either actin, fragmin or Cap42(a)¹. Hence, these four 42,000-MW proteins are functionally distinguishable from each other.

Like actin, Cap42(b) forms a very tight (1:1) complex with DNase I⁴. Consequently this complex is no longer phosphorylated by Cap42(b) kinase. Neither Cap42(a) nor fragmin binds to DNase I. Tight heterodimers form between actin and fragmin as well as between Cap42(b) and Cap42(a)¹⁻⁸. The actin-fragmin interaction is Ca^{2+} -dependent, whereas the Cap42(b)-Cap42(a) interaction is Ca^{2+} -independent. Their interactions appear to be highly specific, because a tight complex is never formed between actin and Cap42(a) or between Cap42(b) and fragmin. These findings indicate the possibility that actin is more closely related to Cap42(b), while fragmin shows a similar relationship to Cap42(a).

To clarify the structural relationships among these four actin-binding proteins, we have studied their immunochemical relations, peptide maps and specific binding to nucleotide triphosphates. The experimental data presented here strongly suggest that Cap42(b), Cap42(a) and fragmin are non-polymerizable but regulatory actin variants. In addition, we provide evidence that pp60^{sarc}, a tyrosine-specific kinase, preferentially phosphorylates fragmin in a Ca^{2+} -dependent manner.

Protein purity

Four distinct actin-binding proteins of 42,000 MW were separately purified from *Physarum* according to the procedures described previously^{1,4,7}. Because all four proteins have approximately the same molecular weight, we carefully tested for a possible cross-contamination in the preparations by various criteria. (1) The two DNase I-binding proteins, actin and Cap42(b), are much more negatively charged than fragmin or Cap42(a)¹, so that in a urea-polyacrylamide gel the former two proteins migrate much faster than the latter two. In our electrophoretic gel system, however, actin and Cap42(b) co-migrate with each other and are not separable unless either of them is phosphorylated selectively by specific kinases. This is also true for fragmin and Cap42(a). (2) A cyclic AMP-dependent protein kinase phosphorylates actin and never any of the three F-actin capping protein preparations⁴. (3) The Cap42(b) kinase from *Physarum* is highly specific for Cap42(b) and in no case phosphorylates any of the other three protein preparations¹. (4) As

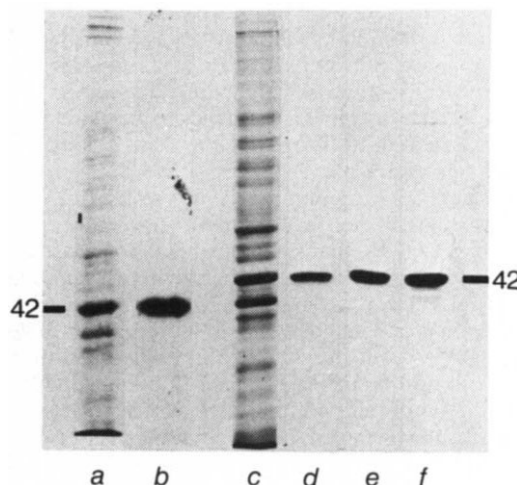


Fig. 1 Immunoblots of whole microplasmodial homogenates of *Physarum* with rabbit antibodies against actin, Cap42(b) and Cap42(a). Proteins in 1.5 μl of packed microplasmodia of *Physarum* were separated by SDS-polyacrylamide (9% or 12%) slab gel electrophoresis, transferred onto nitrocellulose, incubated with four different IgG preparations from rabbits and then reacted with ¹²⁵I-protein A. *a*, *c*, Coomassie blue stained peptides of whole microplasmodial homogenates in SDS-polyacrylamide (9 or 12%) gels. *b*, *d*, *e*, *f*, Autoradiograms of the immunoblots: *b*, IgG against SDS-denatured Cap42(b); *d*, IgG against native Cap42(b); *e*, IgG against SDS-denatured Cap42(a); *f*, IgG against oxidized actin. The marker 42 indicates the molecular weight ($\times 10^{-3}$) of standard peptides.

we described in detail here, pp60^{sarc} preferentially phosphorylates fragmin but none of the other three protein preparations (Fig. 5). (5) Cap42(a) is only able to inhibit almost completely the phosphorylation of Cap42(b) in the presence of actin and Ca^{2+} (ref. 1). (6) The actin sample never showed any significant F-actin capping activity⁴. Using these criteria, which in each case are unique for only one of the four proteins, we could be sure that there was little cross-contamination in any of the four protein preparations used in our experiments.

Immunological cross-reactivity

To examine whether these four proteins share any common antigenic sites, we raised polyclonal antibodies in rabbits against Cap42(a) (SDS-denatured), Cap42(b) (native or SDS-denatured), fragmin (native) and actin oxidized by performic acid⁹ to enhance its immunogenicity. IgGs were purified by affinity chromatography on protein A.

Immunoblots show that IgGs against actin, Cap42(b) or Cap42(a) react almost exclusively with peptides of 42,000 MW in the whole microplasmodial homogenate (Fig. 1). Occasionally anti-actin reacts with two additional peptides of 55,000 and 90,000 MW but much more weakly than with 42,000-MW peptides. The peptide of 55,000 MW might be α -tubulin because a region of α -tubulin has been shown to contain an amino acid

Fig. 2 Immunological reactions of four distinct rabbit antibodies with the four separable proteins of 42,000 MW in *Physarum*. Rabbit IgGs against SDS-denatured Cap42(b) (a), native Cap42(b) (b), SDS-denatured Cap42(a) (c) and oxidized actin (d) were affinity-purified on protein A-Sepharose and their immunological reactions with native actin, Cap42(b), Cap42(a) and fragmin were determined by solid-phase radioimmunoassays (RIAs) including ^{125}I -labelled protein A as a radioactive indicator for IgG. Briefly, 1 μg of antigen in TDSA buffer (10 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol (DTT), 15 % sucrose, 0.02 % NaN_3) was dried onto the surface of an RIA well. After washing with TTX buffer (10 mM Tris-HCl, pH 7.5, 0.1 % Triton X-100, 0.15 M NaCl, 0.1 % bovine serum albumin, 0.02 % NaN_3), the wells were incubated with various dilutions of IgG in the same buffer at 25°C for 1 h. After removal of unbound IgGs by washing with TTX buffer, 0.3 ng of ^{125}I -protein A (25,000 c.p.m. per well) in TTX buffer was added and incubated at 25°C for 1 h. Unbound protein A was then removed by washing with TTX buffer, and the radioactivity of protein A bound to IgG was measured. Although the data are not shown, the rabbit IgG against *Physarum* actin reacted equally well with oxidized and native actin from *Physarum* but not with rabbit skeletal muscle actin.

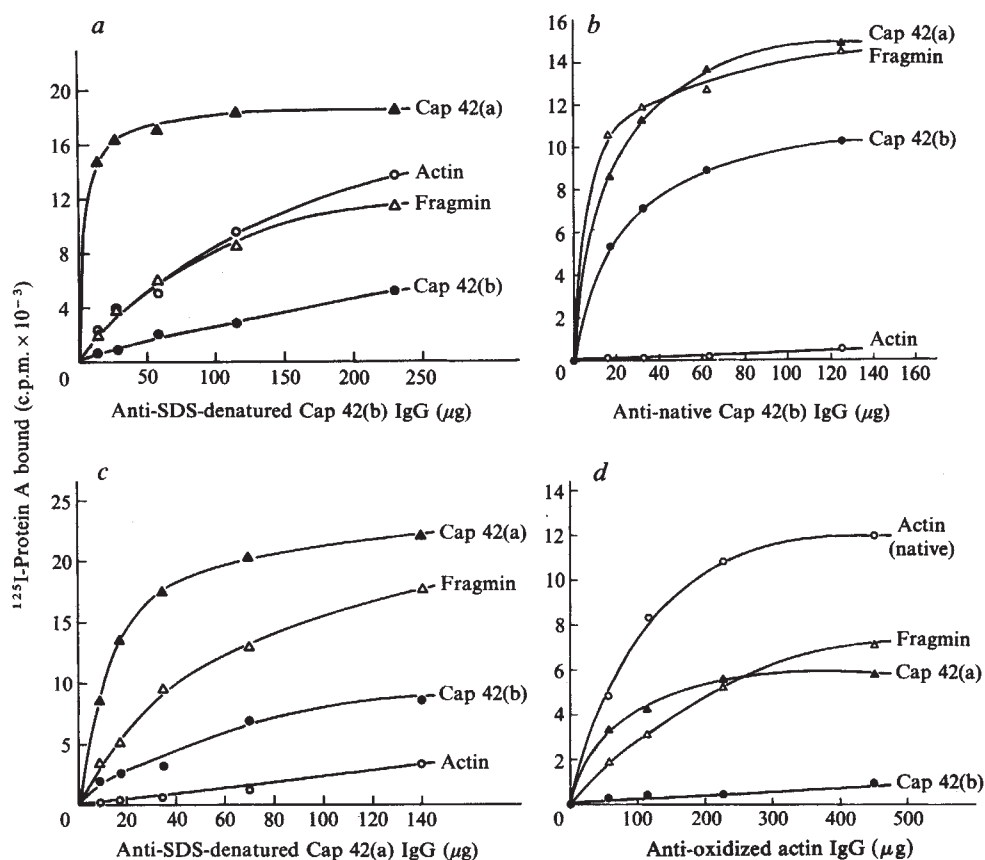


Table 1 Functional differences and sequence homology between four proteins

	Actin	Cap42(b)	Cap42(a)	Fragmin
Polymerization	+	-	-	-
Phosphorylation (Ser)	+	-	-	-
Phosphorylation (Thr)	-	+	-	-
Capping F-actin	-	+	+	+
Severing F-actin	-	-	-	+
Phosphorylation (Tyr)	-	-	-	+
Binding to DNase I	+	+	-	-
Binding to UTP	-	++	+	-
Binding to ATP	+	+	++	+
Binding to IgG I	++	+	+++	++
Binding to IgG II	-	+	++	++
Binding to IgG III	-	+	++	++
Binding to IgG IV	-	+	++	++
Binding to IgG V	++	-	+	+

Phosphorylation either by cyclic AMP-dependent protein kinase from bovine heart⁴ (Ser), or by *Physarum* Cap42(b) kinase¹ (Thr), or by pp60^{src} (Tyr). The abbreviations used for rabbit antibodies are: IgG I, anti-SDS-denatured Cap42(b); IgG II, anti-native Cap42(b); IgG III, anti-SDS-denatured Cap42(a); IgG IV, anti-fragmin; IgG V, anti-oxidized actin.

sequence very similar to that of actin¹⁰. Another as yet unidentified peptide of 90,000 MW obviously cross-reacts with this IgG at least in its denatured form. IgGs against fragmin, affinity purified on fragmin-Sepharose, were shown previously to react only with peptides of 42,000 MW in crude extracts of *Physarum*¹¹.

The cross-reactivity of these antibodies with the four distinct 42,000 MW actin-binding proteins was determined by solid-phase radioimmunoassays (RIAs). Rabbit antibodies against SDS-denatured Cap42(b) react not only with Cap 42(b) but also with actin, Cap42(a) and fragmin (Fig. 2a). Preincubation of this IgG fraction with *Physarum* actin reduces the binding to

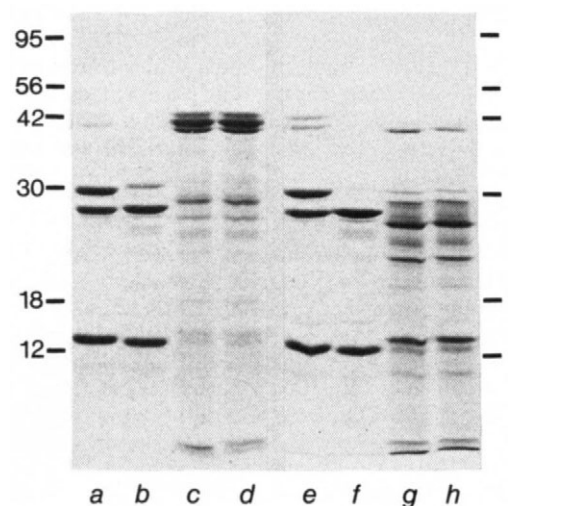


Fig. 3 Tryptic peptide maps of Cap42(b), actin, fragmin and Cap42(a). After boiling in the presence of 0.5 % SDS and 40 mM DTT and a 5× dilution into TDSA buffer, 20 μg of each protein were digested at 35°C for 10 min with 0.1 or 0.4 μg of trypsin, and their tryptic fragment patterns were analysed by SDS-polyacrylamide (16%) gel electrophoresis. a-d, Digested with 0.1 μg of trypsin; e-h, digested with 0.4 μg of trypsin. a, e, Cap42(b); b, f, actin; c, g, fragmin; d, h, Cap42(a). The markers indicate molecular weights ($\times 10^{-3}$) of standard peptides.

all four proteins (data not shown). On the other hand, rabbit antibodies elicited by immunization with native Cap42(b) (Fig. 2b) and SDS-denatured Cap42(a) (Fig. 2c) as well as native fragmin (data not shown) react more strongly with fragmin, Cap42(a) and Cap42(b) than with actin. Rabbit antibodies against oxidized actin from *Physarum* react with native actin from *Physarum* as well as with fragmin and Cap42(a), but not with Cap42(b) (Fig. 2d). These data indicate that actin, fragmin,

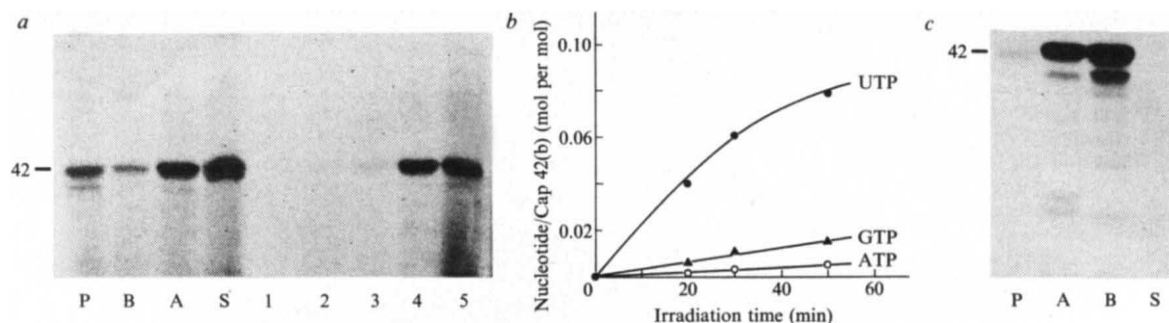


Fig. 4 *a*, Direct photoaffinity labelling by [α - 32 P]ATP of four proteins of 42,000 MW from *Physarum*. 2 μ M actin, Cap42(b), Cap42(a) or fragmin were preincubated with 80 μ M [α - 32 P]ATP (20 μ Ci) in 50 μ l of TDSA buffer at 0 °C for 30 min, and then irradiated by UV light (λ 254 nm) at a distance of 2–3 cm for 30 min according to the procedure described previously²². The radioactive ATP covalently bound to each peptide was separated from free ATP by SDS-polyacrylamide gel (9%) electrophoresis and visualized by subsequent radioautography. Lane P, actin; lane B, Cap42(b); lane A, Cap42(a); lane S, fragmin. Effects of a large excess (10 mM) of non-radioactive ATP and other nucleotide triphosphates as well as diphosphate and monophosphate on the direct photoaffinity labelling of Cap42(a) by [α - 32 P]ATP were also examined under the same conditions and compared with the control *a*: lane 1, ATP; lane 2, GTP; lane 3, UTP; lane 4, pyrophosphate; lane 5, phosphate. Only nucleotide triphosphates almost completely inhibited the covalent binding of radioactive ATP to Cap42(a). *b*, Covalent binding of UTP, GTP and ATP to Cap42(b) by UV irradiation. The time course of the direct photoaffinity labelling of Cap42(b) was followed in the presence of 80 μ M [α - 32 P]UTP, [α - 32 P]GTP or [α - 32 P]ATP at 0 °C for the indicated time by filter paper assay as described previously²². *c*, Only Cap42(b) and Cap42(a) covalently bound UTP by UV irradiation. The direct photoaffinity labelling of Cap42(b) by [α - 32 P]UTP was also compared with those of the other three proteins of 42,000 MW in the same conditions as described for *a*. Lane P, actin; lane A, Cap42(a); lane B, Cap42(b); lane S, fragmin.

Cap42(a) and Cap42(b) share some antigenic sites, that the three F-actin capping proteins have a subset of antigenic sites in common that are not shared with actin and that Cap42(b) lacks some antigenic sites which are commonly present in actin, Cap42(a) and fragmin. Among the three F-actin capping proteins, Cap42(a) and fragmin are very similar to each other in these immunochemical reactions.

Tryptic peptide maps

One-dimensional tryptic peptide maps of Cap42(b) are very similar but not identical to those of actin (Fig. 3), whereas maps of Cap42(a) are almost identical to those of fragmin. The maps of these two pairs of similar proteins differ considerably from each other, suggesting that the four proteins may fall into two groups. One pair is Cap42(a) and fragmin, neither of which binds to DNase I. The other pair, Cap42(b) and actin, both bind to DNase I.

Photoaffinity labelling

Actin has at least one binding site for ATP or ADP¹² and can be labelled with [α - 32 P]ATP by a photoaffinity technique (Fig. 4a). The same treatment covalently labels Cap42(b), Cap42(a) and fragmin (Fig. 4a), with Cap42(a) being the most heavily labelled. The binding of radioactive ATP to Cap42(a) is inhibited almost completely by a large excess (~100 times) of non-radioactive UTP, GTP or ATP, indicating that both UTP and GTP probably share the same binding site on Cap42(a) with ATP. Cap42(b) is most heavily labelled by [α - 32 P]UTP among the three nucleotide triphosphates tested (Fig. 4b). Furthermore, the binding of radioactive UTP to Cap42(b) is inhibited almost completely by a large excess of non-radioactive ATP, GTP or UTP (data not shown), again indicating that these three nucleotide triphosphates probably bind to the same site on Cap42(b). Interestingly, as shown in Fig. 4c, neither actin nor fragmin binds UTP, although both proteins bind ATP (see also Fig. 4a).

Phosphorylation of fragmin by pp60^{sarc}

pp60^{sarc} is a phosphoprotein of 60,000 MW encoded by the *sarc* gene which is solely responsible for the Rous sarcoma virus-induced transformation of avian cells^{13,14}. This protein is associated with a protein kinase activity which phosphorylates preferentially tyrosine residues of several proteins including the heavy chain of IgG, vinculin, casein and pp60^{sarc} itself^{14–17}. Recently a peptide of 42,000 MW called pp42 was shown to be phosphorylated *in vivo* at its tyrosine residues during Rous sarcoma virus-induced transformation¹⁸. pp42 appears to be distinct from any of the polymerizable actin variants, however, as its isoelectric

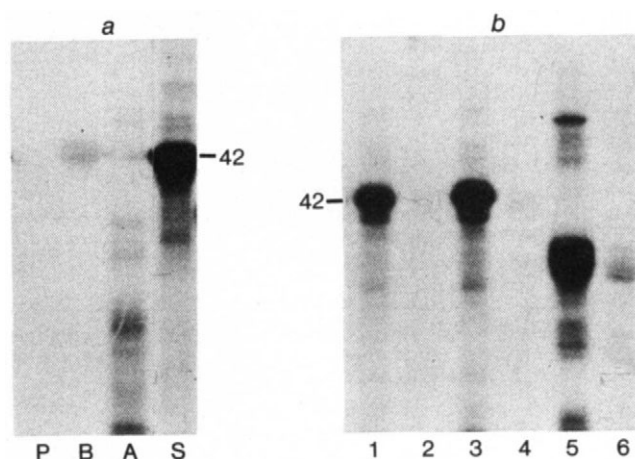


Fig. 5 *a*, Only fragmin was phosphorylated by pp60^{sarc}. 20 μ g of the four proteins of 42,000 MW were separately incubated with pp60^{sarc} at 35 °C for 30 min in the presence of 40 μ M [γ - 32 P]ATP, 10 mM Mg²⁺ and 0.2 mM Ca²⁺. The phosphorylation of each peptide was analysed by SDS-polyacrylamide (9%) gel electrophoresis and subsequent autoradiography. Lane P, actin; lane B, Cap42(b); lane A, Cap42(a); lane S, fragmin. The marker (42) indicates peptides of 42,000 MW. *b*, Ca²⁺ stimulated the phosphorylation of fragmin by pp60^{sarc}. The phosphorylation of fragmin by pp60^{sarc} was carried out in the presence or absence of Ca²⁺, otherwise in the same conditions as described for *a*. Lane 1, fragmin + pp60^{sarc} + 1 mM EGTA; lane 2, fragmin + 1 mM EGTA; lane 3, fragmin + pp60^{sarc} + 0.2 mM Ca²⁺; lane 4, fragmin + 0.2 mM Ca²⁺; lane 5, α -casein + pp60^{sarc} + 0.2 mM Ca²⁺; lane 6, α -casein + 0.2 mM Ca²⁺. Marker 42 indicates fragmin.

point is around 7.0 instead of 5.4 (= *pI* of actin) and its biological activity and the effect of phosphorylation are still unknown. Interestingly, microinjection of Cap42(a+b), an equimolar complex of Cap42(a) and Cap42(b), causes a rapid and reversible disruption of actin stress fibres in mammalian fibroblasts¹⁹ which is similar to that occurring at an early stage of virus-induced transformation of the cells. As pp42 might be a non-polymerizable actin variant, we have asked if pp60^{sarc} is able to phosphorylate some of the three F-actin capping proteins of 42,000 MW from *Physarum*. A preparation of pp60^{sarc} (gift of Drs Y. Sugimoto and R. L. Erikson) was purified to homogeneity from European field vole cells transformed by the Schmidt-Ruppin strain of Rous sarcoma virus (unpublished data). We found that pp60^{sarc} preferentially phosphorylates fragmin in a Ca²⁺-dependent manner (Fig. 5). Neither actin nor Cap42(b),

nor Cap42(a) serve as a substrate for pp60^{sarc}. These results provide additional evidence for our hypothesis that fragmin is functionally distinct not only from actin and Cap42(b) but also from Cap42(a). It remains to be determined, however, whether F-actin capping and severing activities of fragmin are significantly affected by its tyrosine phosphorylation.

Conclusions

Physarum has at least four actin-binding proteins of 42,000 MW; actin, Cap42(b), Cap42(a) and fragmin, that can be distinguished functionally (Table 1), but the cross-reactivity of antibodies, peptide maps and photoaffinity labelling by nucleotide triphosphates provide evidence that the four proteins have common as well as unique structural features (Table 1). We suggest that Cap42(b), Cap42(a) and fragmin are variants of actin that can bind to the fast-growing end of actin filaments but cap the polymer because they cannot propagate further growth. The four proteins fall into two closely related pairs: actin and Cap42(b), and Cap42(a) and fragmin, actin and fragmin share; the fewest common features. Based on all the available data concerning the structure and function of the four proteins, we suggest that they are related to each other in the following order—actin, Cap42(b), Cap42(a), fragmin. Proof of these relationships will be provided by the primary structure of the proteins and their genes. Preliminary amino acid sequence data

(ref. 20 and J. Vandekerckhove *et al.*, in preparation) show that even fragmin has regions identical to actin, although the former has a unique sequence region not shared by the latter. Thus, it seems clear that at least two of these proteins, actin and fragmin, are derived from two distinct actin genes. Similarly, the amino acid sequences of Cap42(b) and Cap42(a) will establish whether they are simply post-translationally modified forms of actin or fragmin, or, as we believe, whether all four proteins are derived from different, distinct genes. Genetic analysis of *Physarum* actin genes suggests that *Physarum* contains at least four and probably five different actin genes²¹. We speculate that these genes code for actin, Cap42(b), Cap42(a) and fragmin and that they were all derived from a common ancestral gene by its duplication and subsequent mutation or rearrangement during evolution of *Physarum*. Finally, because all four distinct 42,000 MW actin-binding proteins in *Physarum* are probably actin variants with different biological activities, we propose to call actin and fragmin by more logical and uniform terms implying both their unique functions and molecular weights. We have called the major polymerizable actin variant Pol 42, and fragmin, an F-actin severing variant, Sev 42.

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LETTERS TO NATURE

Improved limits on small-scale anisotropy in cosmic microwave background

Juan M. Uson & David T. Wilkinson

Physics Department, Princeton University, Princeton, New Jersey 08544, USA

As a remnant of the early Universe, the cosmic microwave background provides unique information on the initial conditions from which matter has evolved to form the structures we see today. All efforts to detect small-scale structure in this radiation have so far been unsuccessful (ref. 1 and refs therein)^{1–4}. Nevertheless, upper limits set on possible underlying fluctuations restrict the range of physical models for perturbations of the density in the early Universe. Our search for small-scale anisotropy in the background radiation has now resulted in a lowering of the upper limit on root-mean-square fluctuations ($\Delta T_{r.m.s.}$) observed at an angular scale of ~ 4 arc min to $\Delta T_{r.m.s.}/T < 2.1 \times 10^{-5}$ at the 95% confidence level (where $T = 2.7$ K, the temperature of the background radiation). The actual limits deduced from our experiment depend on the model assumed for the unseen fluctuations. Several possibilities are discussed as well as the implications this new measurement has for various cosmological models.

The 140-foot telescope of the US National Radio Astronomy Observatory (NRAO), with the K-band maser receiver mounted at the Cassegrain focus, is an excellent instrument for high-

sensitivity continuum observations². This system routinely achieves a temperature of 50 K with 370 MHz bandwidth, centred at 19.5 GHz. Moreover, it is exceedingly stable for long integrations and consistently reaches a sensitivity of $\Delta T_{r.m.s.} = 10^{-4}$ K (antenna temperature) for 2 h of observation in the best weather conditions (clear sky and ambient temperature below 0°C). At this frequency, the half-power beamwidth is about 1.5 arc min.

We have used this system to search for small-scale anisotropy in the microwave background radiation. Although our first observations were made in December 1980, we shall be concerned here only with data collected since December 1982, which are of much better quality than previous data. Good data were obtained from 27–31 December 1982 (82 h) and 16–20 May 1983 (night-time only, 30 h): these have been discussed in ref. 2. The new observations reported here were made between 25 February and 5 March 1984 (88 h) with additional data taken between 3 and 7 January 1984 (4 h) in a filler programme scheduled with other observations. Thus, a total of 174 h of high quality data were obtained from these observing runs out of a total of 445 h scheduled initially.

Fluctuations in the gain of the receiver, as well as variable contributions of radiation from the atmosphere and the ground, are important sources of systematic errors in this kind of measurement. To overcome these problems, the telescope beam is switched at a rate of 3.33 Hz between two positions in the sky, separated by 4.5 arc min, by mechanically oscillating the secondary reflector. The receiver output, δT , which is detected synchronously with beam position, is thus a measure of the difference between the antenna temperatures of the radiation

collected from the two positions in the sky with the addition of a variable offset, due to the differences of atmospheric and ground radiation in the two beams. To eliminate this variable offset, the telescope is periodically moved by 4.5 arc min to place a given sky position (called the field) alternately in the two beams. This gives two sets of data for each field

$$\delta T_{\text{on}} = T_{\text{Field}} - T_{\text{ref2}} + T_{\text{offset-on}} \quad (1)$$

and

$$\delta T_{\text{off}} = T_{\text{ref1}} - T_{\text{Field}} + T_{\text{offset-off}}$$

where T_{ref1} and T_{ref2} correspond respectively to the westerly and easterly reference positions, 4.5 arc min away from the corresponding field. The two offset signals in equation (1) cannot be measured with sufficient accuracy to subtract them; furthermore, they are slowly varying functions of time and telescope position. By accurately timing the off-on observing sequence, one hopes that both offsets will be equal and will cancel in the expression

$$\begin{aligned} \Delta T_{\text{Field}} &= (\delta T_{\text{on}} - \delta T_{\text{off}})/2 \\ &= T_{\text{Field}} - (T_{\text{ref1}} + T_{\text{ref2}})/2 \end{aligned} \quad (2)$$

which is the quantity measured for each field. The atmospheric and ground effects are too complex to predict at the 10^{-4} K level, so one must try and see what happens.

The 12 candidate fields were chosen as close to the north celestial pole as safe operation of the 140-foot telescope allows: declination (1950.0) $86^{\circ}51'$ with right ascensions: 1 h 30 min, 3 h 30 min, ..., 23 h 30 min. This allows 2 h of observation of each field on each day, while minimizing the motion of the telescope with respect to the ground and the atmosphere. At this declination and with a 4.5 arc min beam separation, the telescope goes through the same range of positions with respect to the ground for an off-on pair if the on-scan is started 5 min 3 s after the off-scan. As changing the position of the telescope takes about 15 s, the best cancellation of T_{offset} is achieved with an integration time of 4 min 48 s for each scan. We emphasize that keeping the residual systematic errors below 10^{-4} K requires the atmospheric and ground contributions to be repeatable to better than one part in 10^5 over times longer than the 10 min duration of the off-on sequence. It is remarkable that, with good weather conditions, this requirement is indeed satisfied with this particular telescope.

After correcting the data for the beam efficiency of the antenna (55%) and converting to units of thermodynamic temperature, we compute the mean values $\Delta T_{\text{Field}}^i$ for the observations of each field on each day (i). The associated standard deviations, σ_{Field}^i , are calculated from the scatter among the different off-on measurements (typically 12 on each day), and are an estimate of the statistical uncertainty in the mean, after 2 h of observation. A typical value for data taken with clear sky is $\sigma_{\text{Field}}^i = 0.25 \times 10^{-3}$ K, whereas one expects 0.14×10^{-3} K from system noise alone. The excess noise at this stage is due to a $1/f$ component in the noise power spectrum of the receiver and to the variable components of the atmospheric and ground contributions to δT_{on} and δT_{off} .

The weighted average of the values of $\Delta T_{\text{Field}}^i$ from the different days in which each field was observed yield the final values $\overline{\Delta T}_{\text{Field}}$ plotted in Fig. 1. The errors associated with each point correspond to the standard deviations, σ_{Field} , estimated from the measurement statistics in the usual way. It is crucial to estimate accurately the errors in Fig. 1 because the data will be used to decide whether a significant sky signal has been detected or, alternatively, at what level a sky signal is excluded. A major concern is whether $\Delta T_{\text{Field}}^i$ changes from day to day owing to slow changes in $T_{\text{offset-on}} - T_{\text{offset-off}}$. This can be checked by comparing the measured day-to-day scatter in $\Delta T_{\text{Field}}^i$ with the corresponding values of σ_{Field}^i . Figure 2 shows a histogram of the weighted residuals $(\Delta T_{\text{Field}}^i - \overline{\Delta T}_{\text{Field}})/\sigma_{\text{Field}}^i$ for the 87 2-h observations. The histogram matches the gaussian distribution expected if all the day-to-day scatter were due to fluctuations

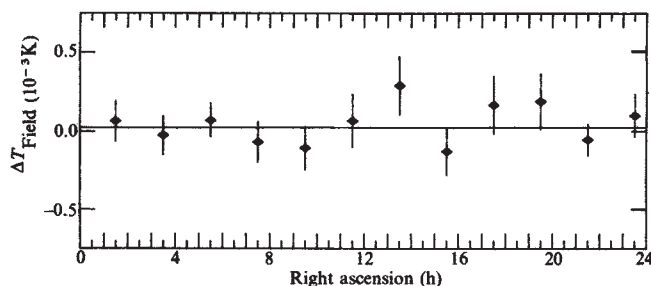


Fig. 1 Final averages for the observed fields. The solid line indicates the overall weighted average, which is $\Delta T_{\text{ave}} = (26 \pm 39) \times 10^{-6}$ K. $\overline{\Delta T}_{\text{Field}}$ is the difference in thermodynamic temperature between the field and the average of two reference positions, 4.5 arc min away on opposite sides. (See equation (2).)

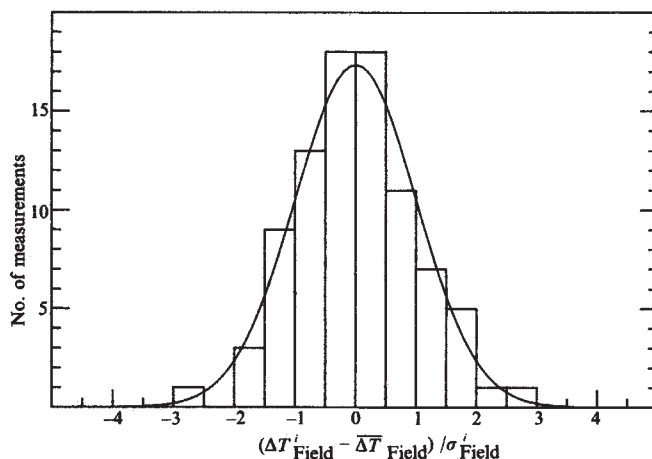


Fig. 2 The day-to-day scatter in measurements averaged over ~ 2 h. As the residuals from the field averages $\Delta T_{\text{Field}}^i$ are used, the possible sky signals have been removed. If there were no extraneous day-to-day effects, the histogram would fit the gaussian curve shown; it does so reasonably well.

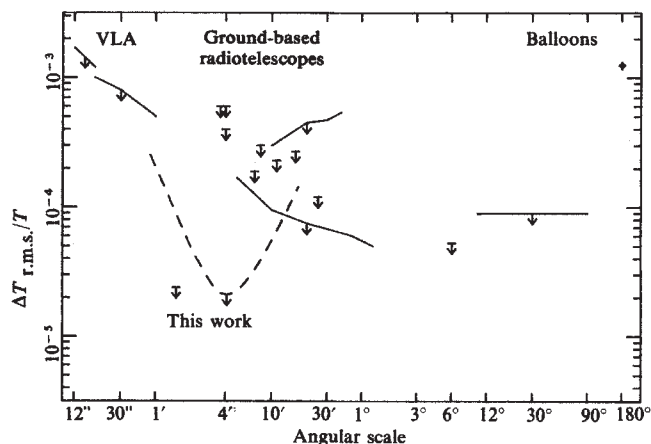


Fig. 3 ∇ , Upper limits (95% confidence level) on anisotropy of the 2.7 K radiation at various angular scales. The Very Large Array (VLA) results^{3,4} are new and will probably be improved. Some of the points between $1'$ and $1''$ have been revised¹ from the values given in the original references. Anisotropy at large angular scales⁸ must be measured from balloons or satellites because of atmospheric noise.

contained in σ_{Field}^2 . Indeed, the corresponding χ^2 is 78 with 75 degrees of freedom. Therefore the values of σ_{Field} displayed in Fig. 1 are not contaminated by day-to-day effects and are accurate estimates of the measurement errors.

The weighted average of the values in Fig. 1 is $\Delta T_{\text{ave}} = (26 \pm 39) \times 10^{-6}$ K. As none of the 12 points differs significantly from this value, no anisotropy has been detected at a level of $\sim 2\sigma_{\text{Field}}$. A more stringent limit can be set by using all the data in a statistical argument, although this requires adopting a model for the anisotropy. The underlying sky signal is expected to be gaussian, distributed between the 12 field positions (separation $> 1.6^\circ$), and can be characterized by its standard deviation, σ_{sky} . Assuming there is no correlation between the signals arriving from a given field and associated reference positions (4.5 arc min scale), the r.m.s. residual in ΔT_{Field} due to a true sky signal is (from equation 2)

$$\sigma_{\text{r.m.s.}}^2 = (4\sigma_{\text{sky}}^2 + \sigma_{\text{sky}}^2 + \sigma_{\text{sky}}^2)/4 = 1.5\sigma_{\text{sky}}^2 \quad (3)$$

In this case, the off-on procedure is a probe of σ_{sky} with $\sqrt{1.5}$ efficiency. This efficiency factor depends on the angular autocorrelation that is assumed for the possible sky signal and must be evaluated separately for each different model.

The Neyman-Pearson lemma prescribes the optimal statistic for testing a hypothetical value of σ_{sky} . In our case the best statistic is essentially a weighted χ^2 (ref. 2), which yields

$$\sigma_{\text{r.m.s.}} = (0.1 \pm 4.7) \times 10^{-5} \text{ K} \quad (4)$$

and an upper limit

$$\sigma_{\text{r.m.s.}} < 7.8 \times 10^{-5} \text{ K (95\% confidence level)} \quad (5)$$

Using equation (3) and $T = 2.7$ K gives

$$\sigma_{\text{sky}}/T < 2.4 \times 10^{-5} \text{ (95\% confidence level)} \quad (6)$$

Limits on anisotropy from different experiments are difficult to compare. Because various scanning patterns and statistical methods have been used, careful statements of limits on a particular model should examine each measurement separately. Figure 3 shows the present experimental limits, most of which assume that the underlying anisotropy is uncorrelated gaussian noise. For this assumption the result of equation (6) applies at an angular scale of 1.5 arc min.

A more general way to interpret our result is to take the Fourier transform of the scan pattern (three gaussian beams 1.5 arc min across, separated by 4.5 arc min) and then to average it over the orientation angle. Thus, the limit shown in Fig. 3 (dashed line) assumes that all of the anisotropy is the result of gaussian sinusoidal fluctuations of a certain scale. The strongest limit corresponds to a scale (half wavelength) of 4 arc min and is

$$\Delta T_{\text{r.m.s.}}/T < 2.1 \times 10^{-5} \text{ (95\% confidence level)} \quad (7)$$

which is more stringent than the one given in equation (6) because the sinusoidal fluctuations are anticorrelated on the scale of our beam-throw, increasing the efficiency of the observing procedure for this kind of fluctuation.

A physical interpretation of the results requires a model for the nature of the underlying fluctuations. Most authors assume fluctuations with a spectrum of the form $(\delta\rho/\rho) \propto k^n$, for the dominant contribution to the mass of the Universe at the time of decoupling of matter and radiation (a redshift of $\sim 1,000$). The amplitudes of the fluctuations are fixed by matching the evolved autocorrelation function of the matter to the data available from studies of galaxy clustering. The details of the interaction between the mass components and the background radiation determine the anisotropy required by each particular model. Comparing our results with recent calculations⁵ rules out those models in which the initial fluctuations are adiabatic and dominated by baryonic matter. Baryon-dominated universes in which the initial perturbations are isothermal are currently out of fashion but are not seriously constrained by our limit: for $n < 0$ one needs to satisfy $0.1 < \Omega < 0.3$ (Ω is the ratio of the density of the Universe to that of an Einstein-de Sitter universe),

whereas no restriction is imposed if $n \geq 0$. Models dominated by massive neutrinos require $\Omega \sim 1$, although they suffer other problems^{6,7}. More exotic possibilities are models in which the dominant component is in the form of yet undiscovered cold, dark matter, such as axions or photinos. These models require that $\Omega \cdot H > 0.2$ (H is Hubble's constant in units of $100 \text{ km s}^{-1} \text{ Mpc}^{-1}$).

Finally, we must caution that the analysis of our data rests on the assumption that the underlying fluctuations have a gaussian distribution: if untrue, the theoretical predictions could be uncertain by factors of 2. Although there is a conflict between the theoretical models and the experimental data, the fate of the various models is still far from settled.

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Effects of pulsation and mass loss on stellar evolution

L. A. Willson & G. H. Bowen

Astronomy Program, Physics Department, Iowa State University, Ames, Iowa 50011, USA

There is much evidence that many post-main sequence stars are losing mass, that the integrated mass loss is sufficient to affect their evolution and that epochs of substantial mass loss are normal for almost all stars. However, neither the precise timing, nor the mechanisms, nor the full consequences of this mass loss are yet well understood. Stellar pulsation may play a key role: it is closely associated with evolutionary phases where substantial mass loss occurs, and there are good physical reasons to expect pulsation to cause, or at least greatly to enhance, mass loss. Here we point out that there are many possible consequences, some not previously recognized, of pulsation-related mass loss for the evolutionary behaviour of post-main sequence stars. We advance the hypothesis that most mass loss of evolutionary significance is closely related to stellar pulsation.

We have been modelling the atmospheric dynamics of Mira variables, which exhibit long-period, large-amplitude radial pulsation and are known to have high mass-loss rates. Recently, we have begun using a similar approach to model other pulsating giants. There are several ways in which pulsation can lead to mass loss, some relevant to Mira variables and some to other classes of stars. Indeed, it now appears almost inevitable that pulsation not only can but does lead to significant mass loss in almost every case, through transfer of momentum and energy to the outer atmosphere. Steady pulsation of even moderately large amplitude generates shock waves and causes great extension of the stellar atmosphere, thereby strongly enhancing all mass-loss processes. Radiative cooling, which depends on collisions, becomes so slow in the low-density outer atmosphere (now a very large region with considerable total mass) that dynamic processes there become effectively adiabatic¹. Non-radiative energy transfer to the outer atmosphere by pulsation-induced shocks can thus have an important effect. In RR Lyrae

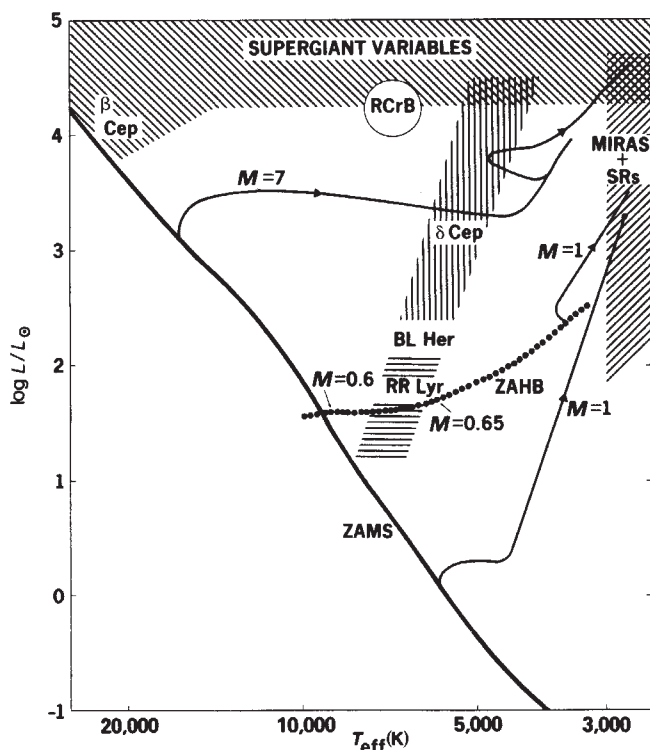


Fig. 1 Hertzsprung-Russell diagram (luminosity versus temperature) showing the most important regions of pulsational instability. Standard zero-mass loss evolutionary tracks of mass equal to $1 M_{\odot}$ and $7 M_{\odot}$ stars of solar composition are sketched in for reference, as well as the zero-age horizontal branch for core He burning stars^{4,5,9}.

models, for example, it heats the outer atmosphere sufficiently to produce a hot corona and cause a substantial coronal wind. In contrast, the outer atmospheres of Mira models remain cool; dust grains form there and the density in the condensation region is increased by many orders of magnitude compared with static models; dust effects dominate and radiation pressure on the grains drives strong winds similar to those observed. With modest changes in stellar parameters, this Mira-like behaviour grades smoothly into models with optically thick circumstellar dust shells and still more massive winds, simulating the infrared OH-IR sources.

Prodigious mass loss must occur before or during the ascent of the asymptotic giant branch, because white dwarf stars, with masses typically of $0.6\text{--}1.0 M_{\odot}$, are formed from progenitors with masses up to 5 or $6 M_{\odot}$, or more^{2,3}. Attempts to reproduce observational evidence, including not only direct comparisons of calculated and observed properties of asymptotic giant branch stars but also the effects of nucleosynthesis and mass recycling on galactic chemical evolution, indicate that the mass-loss process must be discontinuous, with a terminal 'superwind' stage removing at least the last few tenths of a solar mass of envelope material in a time which is short on the nuclear evolutionary time scale of these stars². This has a natural explanation, supported by observed mass-loss rates, in terms of rapid pulsation-induced mass loss during the brief but very common Mira stage of evolution at the tip of the asymptotic giant branch⁴.

Calculations indicate that the evolutionary track of helium core-burning (horizontal branch) stars with $M \sim M_{\odot}$ will lie to the red of the RR Lyrae instability strip; only stars with low metallicity and/or masses $\leq 0.7 M_{\odot}$ evolve far enough into the blue during this stage to produce RR Lyrae stars^{5,6}. Most of this mass is presumably lost during the first ascent of the star up the red giant branch. Near the end of this ascent, these stars probably enter the same pulsational instability region which the Mira and semiregular variables occupy at higher luminosity. A reasonable hypothesis is that pulsation near the tip of the red

giant branch provides the mechanism for mass loss needed to produce RR Lyrae stars.

Mass loss on the horizontal branch may also be indicated. Comparison of evolutionary calculations for horizontal branch stars with the observed blue-red horizontal-branch populations, and with the statistics of RR Lyrae stars, further indicates that within a given cluster there must be a modest range of masses along the horizontal branch: $\sim \pm 0.02 M_{\odot}$ (refs 5, 6). This has been assumed to be owing to stochastic variations in the mass loss on the red giant branch, but it can equally well be accounted for by assuming pulsation-induced mass loss during the RR Lyrae stage. As horizontal branch evolution is relatively slow, the mass-loss rates required are undetectably low at present: $\sim 10^{-10} M_{\odot} \text{ yr}^{-1}$ is sufficient to produce the range in masses needed to populate the blue and red horizontal branches of clusters.

This rate of mass loss from RR Lyrae stars has further implications for the morphology of cluster Hertzsprung-Russell (H-R) diagrams and for the ultimate fate of low-mass stars. One possible consequence of mass loss due to pulsation in the RR Lyrae stars is the trapping of these stars along the edges of the instability strips for fundamental and overtone pulsation. For those RR Lyrae stars evolving to the right in the H-R diagram, the effect of a decrease in mass is to force them to the left—possibly cancelling the evolutionary effect. If such a star were to tend to evolve into a region of the H-R diagram where, owing to its pulsation properties, its mass-loss rate would be large, it may become trapped by these competing effects at the blue boundary of the region. If the mass loss during overtone pulsation (which takes place at the blue side of the instability strip) is too low to inhibit evolution to the red, but the fundamental mode mass-loss rate is larger, the star may be trapped where the mode change occurs. This trapping would have an effect on the period distribution of RRc and RRab stars similar to the 'hysteresis mechanism' proposed by von Albada and Baker⁷ and further elucidated by Stellingwerf⁸. In addition, trapping in the mode-switching region could explain the overabundance of double-mode RR Lyrae stars in clusters such as M15 (ref. 9). This trapping effect has as a further consequence, that quite low rates of mass loss are given more time to operate, thus producing greater net mass loss than would otherwise be supposed.

Mass loss during the horizontal branch pulsation stages should have observable effects on the RR Lyrae period distribution, on the luminosity difference between RR Lyrae and BL Her stars in the same cluster and on the relative population of the red and blue horizontal branches. Pulsation masses for the post-RR Lyrae BL Her stars are less than those for RR Lyrae stars¹⁰, as would be expected from our picture. The alteration of horizontal branch (HB) evolution by RR Lyrae pulsation-induced mass loss suggests the possible existence of a post-HB stage of pre-white dwarfs, which could explain the great blueward 'tail' of some cluster HBs. This would also lead to the formation of a separate group of low-mass ($\sim 0.5 M_{\odot}$) white dwarfs of high He content, from those stars whose He burning stage was terminated prematurely by RR Lyrae stage, pulsation-driven mass loss. Such post-HB stars may have been observed in the He shell-burning hot O subdwarfs, studied by Schonberner and Drilling¹¹.

Another form of trapping may be operating in the Cepheids, and may account for part of the persistent 'mass anomaly' between evolutionary and pulsationally derived (pulsation, beat and bump) masses^{12,13}. For shorter period Cepheids, models calculated on the assumption that mass loss on the red giant branch has removed enough mass before the Cepheid stage to reduce the masses to the 'beat' and 'bump' masses do not loop to the blue far enough to enter the instability strip. However, if the mass loss starts only when the star is already in the strip, this difficulty is removed. Furthermore, the stars spend most of their time near the blue end of the blue loops¹³. Mass loss during Cepheid pulsation may therefore have the effect of bringing this long-lived stage into the instability strip, allowing more time for the stars to lose mass. Observed mass-loss rates for at least some

Cepheids are in the range required if trapping operates: infrared excesses imply mass-loss rates of $\sim 10^{-6} M_{\odot} \text{ yr}^{-1}$ for SV Vul (period 45 days) and RS Pup (42 days); RS Pup appears to be surrounded by five shells of ejected material implying considerably higher mass-loss rates in the recent past^{14,15}. For the shorter period Cepheids, where the mass discrepancy is most serious, circumstellar lines indicative of material moving out with velocity $v > v_{\text{esc}}$ have been seen in the ultraviolet¹⁶.

Although various authors have argued that the Cepheid mass discrepancy lies entirely with the pulsation models, this conclusion depends on adopting a particular distance scale, for example, one with distance modulus ($m - M \geq 18.7$) for the Large Magellanic Cloud. If, as recent studies suggest¹⁷, the LMC distance modulus is nearer 18.5, the discrepancy between evolutionary and pulsation masses returns. Thus, for example, Schmidt¹⁸ finds only the evolutionary mass to be discrepant on his preferred distance scale.

There are many other known links between pulsation and mass loss. For example, we find pulsations reported for the heavily mass-losing R CrB class stars¹⁹; pulsations invoked to account for the large mass-loss rates of the Wolf-Rayet stars²⁰; infrared excesses detected for the pulsating RV Tauri stars¹⁴;

and variability and mass loss observed for the luminous supergiants, with a correlation between the amplitudes of variation and the mass-loss rates²¹.

The hypothesis that most, if not all, evolutionarily significant mass loss occurs as the direct or indirect result of stellar pulsation promises to resolve some long-standing problems in stellar evolution theory. It is a hypothesis which may be relatively easy to incorporate into future theoretical calculations, as the locations of regions of pulsational instability in the H-R diagram are well known. As in this hypothesis mass loss occurs quite rapidly during short episodes for most stars, evolutionary changes in the stellar parameters during such episodes will be small, allowing at least a partial separation of the mass-loss and evolutionary calculations. Efforts to observe and measure mass loss from pulsating variables as a function of their pulsation properties should provide further constraints on the mass-loss process.

We are continuing our investigations into the mechanisms and rates of mass loss caused by pulsation, for different types of stars using detailed numerical hydrodynamics. Here we have attempted only to give a brief and qualitative description of some of the major effects of pulsation in stellar systems. A full report on the methods and results will appear elsewhere.

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Observations and theory of the solar semidiurnal tide in the mesosphere of Venus

Stephen B. Fels*, J. T. Schofield†‡ & David Crisp*‡

* Geophysical Fluid Dynamics Laboratory/NOAA, Princeton University, Princeton, New Jersey 08542, USA

† Department of Atmospheric Physics, University of Oxford, Oxford OX1 3PU, UK

The Orbiter Infrared Radiometer experiment, aboard the 1978 Pioneer Venus mission, produced strong evidence for the existence of a semidiurnal solar tide in the mesosphere of Venus¹. At the same time, measurements *in situ* of radiative fluxes and cloud particle distribution, in other Pioneer Venus experiments, have provided much information about the structure of the solar heating and infrared cooling there²⁻⁴, making it possible to calculate the thermal tidal forcing functions with confidence, and also allowing good estimates of the radiative dissipation rates to be made. Because the structure of tidal fields depends sensitively on that of the background zonal mean wind velocity, $U(\theta, z)$, a knowledge of the sources and sinks of tidal energy and of the actual structure of the tide should allow one to infer the behaviour of U . We show here that a physically reasonable mean flow can be found that leads to theoretically predicted tides that are in excellent agreement with those observed. Our zonal mean flow shows a maximum velocity of $\sim 130 \text{ ms}^{-1}$ at 70 km, decreasing dramatically to ~ 0 at 88 km, and a jet-like meridional structure that deviates strongly from solid-body rotation.

The observed semidiurnal thermal tide is taken to be the solar-fixed, wavenumber-two component of the temperature field derived from the Orbiter Infrared Radiometer observations. This instrument made measurements in five different channels,

with vertical response or weighting functions peaked at about 99, 86, 76, 68 and 64 km for channels 1 through 5 respectively. We are particularly interested in the region 70-90 km (channels 2, 3 and 4), as below this the measurements are complicated by clouds, and above it the theory loses its validity, owing to the weakening of the basic-state zonal wind and the importance of radiative damping. Vertical resolution is limited by the 10-km width of the weighting functions—a fact of great importance in interpreting any theoretical results. Hence, to compare theory and observation, we have used the tides predicted by the model to generate synthetic radiances, and have compared these with the measurements. Orbiter Infrared Radiometer radiances for each channel, accumulated over 72 days, have been binned in a solar-fixed, latitude-longitude, zenith-angle grid¹. Figure 1 displays channel 2, 3 and 4 semidiurnal amplitudes and phases, derived from a Fourier analysis of this grid, as a function of latitude for two zenith angles. Beyond 65°N, strong short and long term variations in atmospheric structure may introduce wavenumber-two features in the time-averaged radiances, whose interpretation as tides is therefore questionable^{1,5}.

Several features appear in the data: (1) The phase increases with height. (2) The amplitude in channel 3 is essentially constant in the meridional direction between 0° and 40°, and then decreases strongly. Similar, though less dramatic, behaviour is seen in channels 2 and 4, which are somewhat more strongly peaked in the tropics. (3) The largest amplitudes are those in channel 3, although classical tidal theory predicts a strong growth of amplitude with height. (4) There is an increase of phase with latitude in channels 2 and 3.

In the Venus mesosphere, the dominant zonal flow is the 'four-day wind'⁶, an extremely strong super-rotation. Therefore, unlike the terrestrial and martian cases, the assumption of an atmospheric mean state represented by a spatially constant angular velocity Ω in inertial space is unsuitable. As a result, 'classical' tidal theory⁷ is not applicable: in particular, the linear tidal equations are no longer separable, and it is in general necessary to resort to multi-dimensional numerical methods for their solution⁸. In the special case, however, where the zonal wind is a slowly varying function of height, an approximation

‡ Present addresses: Jet Propulsion Laboratory, California Institute of Technology, Pasadena, 91109, USA (J.T.S.); Division of Geological and Planetary Sciences, California Institute of Technology, Pasadena, California 91125, USA (D.C.).

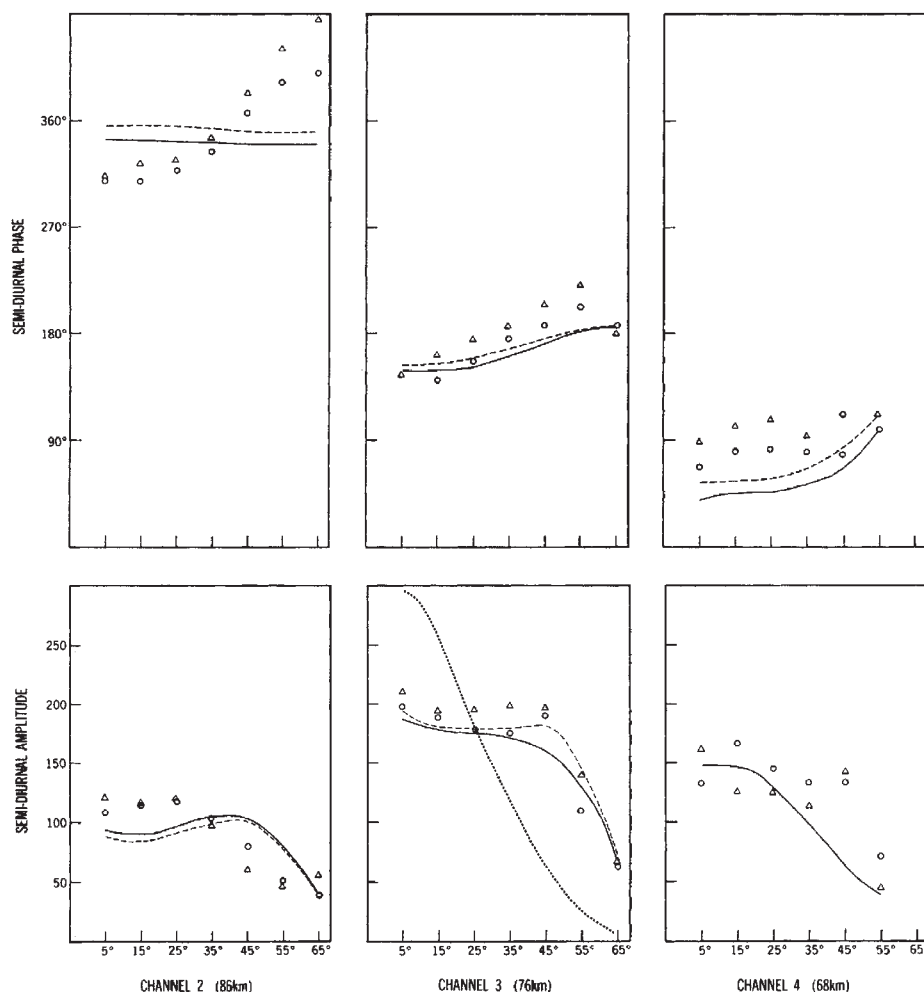


Fig. 1 Observed and predicted semidiurnal radiances. The observed mean amplitudes and phases in each of channels 2, 3 and 4 are shown for two different zenith angles (41° , $\circ$; 57° , Δ). The theoretical predictions for these two zenith angles are the solid and broken curves respectively. The amplitudes are in units of $\text{nW cm}^{-2} \text{sr}^{-1} \text{cm}$, and the phase convention is that of ref. (1). This is a representative sample of the information available for seven different zenith angles. As explained in the text, poleward of about 65° , the observations are ambiguous, and are therefore not shown. The dotted line in the amplitude diagram for channel 3 shows the meridional structure the tide would have for solid-body rotation.

can be used to preserve much of the simplicity of the classical case (S. B. Fels, unpublished work). This method, which is similar to the Wentzel-Kramers-Brillouin-like techniques developed by others^{9,10}, leads to the description of the tidal geopotential height field $\Phi'(\phi, z, \mu)$ as

$$\Phi' = e^{(2i\phi + z/2H)} \sum_n f_n(z) Y_n(\mu, z) \quad (1)$$

Here $z = -H \log(P/P_0)$, H is a reference scale height calculated at temperature T_0 , ϕ is the longitude, P the pressure, P_0 the surface pressure, and μ the sine of the latitude. The partial differential equation governing Φ' can then be treated as though it were separable, with no coupling between the various modes.

The horizontal structure functions Y_n are generalizations of the usual Hough functions, and satisfy the eigenvalue equation

$$\frac{1}{\omega(\mu, z)} \left\{ \frac{\partial}{\partial \mu} \left[\frac{1-\mu^2}{D} \frac{\partial}{\partial \mu} - \frac{2\mu}{D} \right] + \frac{1}{D} \left[\left(2\mu - \frac{1-\mu^2}{\omega} \frac{\partial \omega}{\partial \mu} \right) \frac{\partial}{\partial \mu} - \frac{4}{1-\mu^2} \right] \right\} Y_n = -\varepsilon_n^2(z) Y_n \quad (2)$$

at each height z . Note that $\omega(\mu, z) = \Omega(\mu, z)/\Omega(0, z)$. By assumption,

$$\frac{dY_n}{dz} / Y_n \ll \frac{df_n}{dz} / f_n$$

The vertical structure functions satisfy the simple second-order ordinary differential equation

$$\left(\frac{\partial}{\partial z} - \frac{1}{2H} \right) \left[\frac{1}{N^2} \left(\frac{\partial}{\partial z} + \frac{1}{2H} \right) f_n \right] + \frac{\varepsilon_n^2}{a^2 \Omega_c^2(z)} f_n = \frac{R}{2iH} \left(\frac{\partial}{\partial z} - \frac{1}{H} \right) \frac{1}{N^2} \int d\mu Y_n(\mu, z) J(\mu, z) / \Omega(\mu, z) \quad (3)$$

Here $N(z)$ is $T_0/T(z)$ times the Brunt-Vaisala frequency, which is based on the observed equatorial static stability, and is independent of latitude; Ω_c , the mean-state angular velocity at the equator; a , the planetary radius; R , the reduced gas constant; J , the tidal heating rate. Below 85 km, the contribution of the higher modes to the sum in equation (1) is small, and so for simplicity we will retain only the gravest mode in our numerical calculations.

The meridional structure of Y_n depends strongly on that of the normalized angular velocity $\omega(\mu, z)$, and so contains information about the behaviour of U in the horizontal direction. The vertical wavelength of the tide is roughly $\Omega_c a / N \varepsilon_n$, so that this quantity provides a measure of the meridionally averaged zonal velocity. In the models we have examined, the gravest semidiurnal tide has a vertical wavelength of 25 km or less in the region of interest.

The tidal heating J is derived by Fourier analysis from the results of a detailed radiative model of the Venus mesosphere¹¹. This model, whose opacity sources include CO_2 , SO_2 and H_2O , as well as cloud particles, was constrained by use of Pioneer Venus observations. The results show that there is an important region of excitation near 64 km, due to the strong absorption of ultraviolet and near-infrared solar radiation by the cloud particles in that region (Fig. 2). A less important heating region due to the near infrared CO_2 bands appears above 80 km.

Damping of the tides by thermal infrared transfer⁸ becomes important whenever the radiative decay time $\tau_{\text{rad}} < \Omega^{-1}$. This is generally true above 83 km in our models and has been taken into account, by modifying equations (2) and (3), to allow for the effects of newtonian cooling. The required relaxation rates (Fig. 2) were taken from ref. (11), and are those appropriate for a disturbance of vertical wavelength ~ 25 km. (They are approximately twice those appropriate for an infinite wavelength case.)

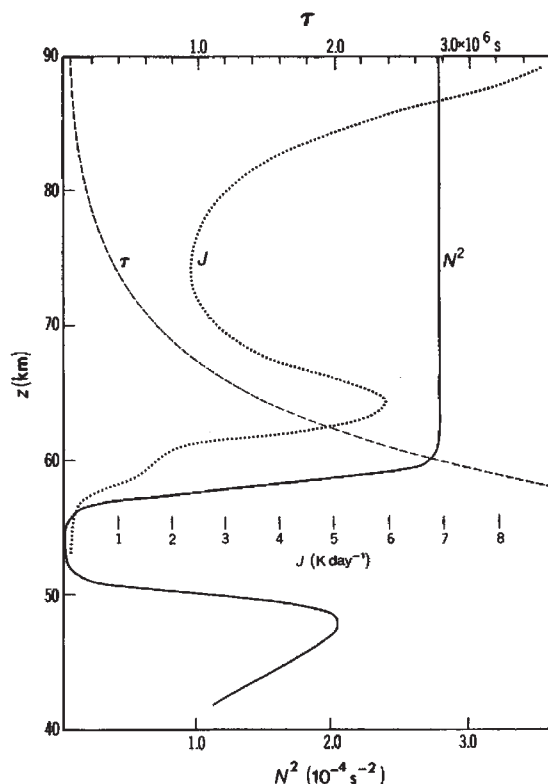


Fig. 2 Vertical structure of the model atmosphere from 40–90 km. The solid line is N^2 , the Brunt-Vaisala frequency squared, in units of s^{-2} (bottom scale); the dotted line is J , the semidiurnal component of the solar heating function at the Equator in degrees K per Earth day (middle scale); the chained line is τ_{rad} , the radiative damping time in seconds (upper scale).

Although the approximations used in reaching this formulation may not be strictly valid, the extreme simplicity of the method and the ease with which it allows for interpretation make it very useful in preliminary studies like this. The simplicity of the theoretical model makes it possible to test many different forms for U . We always demand that U , at 60 km, be close to the form deduced from Pioneer Venus ultraviolet cloud observations¹², which imply an equatorial velocity of $\sim 100 \text{ ms}^{-1}$, and a rotation profile which is close to solid-body.

Figure 3 shows the structure of U that best fits the tidal observations; the resulting predictions are given in Fig. 1. The agreement is seen to be very good in general. The most noticeable errors occur in channel 2, where the latitudinal structure of the amplitude and phase is not well reproduced. This is probably owing to the inclusion of only one mode, whose structure does not match that of the local heating at 90 km. At this level and above, $\Omega\tau_{\text{rad}}$ is small, and the thermal structure depends largely on the local heating. The errors in the amplitude and phase in channel 4 are possibly owing to incorrect specification of the details of the heating function J .

The following considerations support our choice of U : (1) Solid-body rotation results in tidal fields whose meridional structure is much too strongly peaked near the equator. To match the observations, especially those of channel 3, it is necessary that Ω be much larger at 50° than at the equator. This has the additional desirable effect of increasing the vertical wavelength of the tide between 70 and 86 km. Without this, and without the region of large U between 67 and 71 km, the phases of channels 2 and 3 would be much too large.

(2) The existence of the overall $e^{z/2H}$ term makes it extremely difficult to keep the channel 2 amplitude below that of channel 3, unless U decreases dramatically with height above 78 km. This has the effect of reducing the parameter $\Omega\tau_{\text{rad}}$ so that damping becomes effective. Also it reduces the vertical

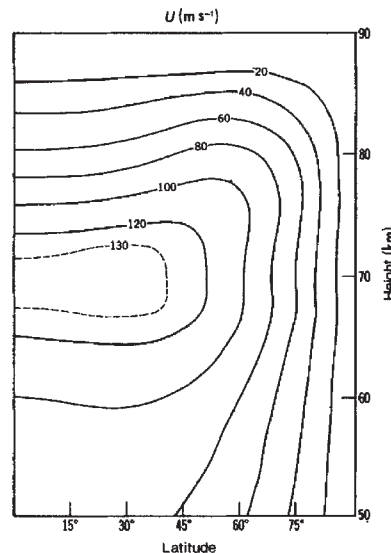


Fig. 3 Vertical and meridional structure of $U(\theta, z)$, the zonal mean wind used in the theoretical tidal model.

wavelength near 88 km, so that the averaging effect due to the thickness of the channel 2 weighting function becomes important. The same conclusion has been reached independently by Pechmann⁸.

The meridional variation in the phase of the tide is largely owing to the effect of radiative damping which destroys the modal character of the horizontal eigenfunction, and allows some meridional propagation to occur.

Whereas it is possible to find other mean-state U structures which give equally good tidal predictions, they all share the chief characteristics of that displayed here. They are also in general agreement with those deduced by Limaye (S. S. Limaye, paper presented at Venus Int. Ref. Atmos. Workshop, Hamburg, 1983) on the basis of cyclostrophic balance with the measured zonal mean temperature, but differ in that the balanced winds are considerably weaker in the tropics than are ours. However, such diagnostic calculations are not without problems¹³, and particularly so in the tropics. The tidal calculations, on the other hand, do not share this deficiency. Indeed, the tidal temperature perturbations based on Limaye's U disagree with observations in two respects: they are too strongly peaked at mid-latitudes and their channel 2 and 3 phases are too large in the tropics by 50° . This is true even when U in the tropics is held fixed at its value at 30° . If, as Limaye suggests, U decreases towards the equator, these failures would be even more pronounced. If, on the other hand, U were to increase equatorward of 30° (S. S. Limaye and A. J. Kliore, preprint), the agreement with observed tides would be better.

The form of U which we propose is not only broadly consistent with observations^{13,14}, but also with theoretical speculation on the role the tides themselves have in decelerating the mean flow above the forcing region¹⁵. In addition, conclusions quite similar to ours have recently been reached by P. J. Valdes (personal communication) using a two-dimensional numerical tidal model. Although this increases confidence in our results, their confirmation must await further observations.

This work was begun while one of us (S.B.F.) was a visitor in the Department of Atmospheric Physics, Oxford University. We thank Drs D. G. Andrews and F. W. Taylor for useful discussions, and Mr P. J. Valdes for showing us his calculations before their publication.

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Comparative utility of microwave and shortwave satellite data for all-weather charting of snow cover

David Robinson*, Klaus Kunzi†, George Kukla* & Helmut Rott‡

* Lamont-Doherty Geological Observatory of Columbia University, Palisades, New York 10964, USA

† Institut für Angewandte Physik, Universität Bern, 3012 Bern, Sidlerstrasse 5, Switzerland

‡ Institut für Meteorologie und Geophysik, Universität Innsbruck, Schopfstrasse 41, A-6020 Innsbruck, Austria

Charting snow cover by shortwave imaging requires visual analysis which is complicated by cloud coverage and poor surface illumination^{1,2}. Microwaves, however, being almost unaffected by clouds and independent of solar illumination, are potentially useful for monitoring the extent and variation in snow cover, in climatological and hydrological studies. Data from spaceborne passive microwave sensors have previously been used to chart regional overland snow cover³⁻⁵; but charting hemispherical snow coverage was not feasible until high spatial resolution and multiple channels were combined in the Scanning Multichannel Microwave Radiometer (SMMR) launched in 1978 on the Nimbus-7 satellite. Here we compare SMMR data with shortwave images obtained over Asia to justify further use of microwave sensors in automated charting of seasonal snow cover under all weather conditions. Agreement between the two methods is found in ~75% of the tested grid points.

Microwave radiation emitted from the Earth's surface is detected by the Scanning Multichannel Microwave Radiometer at five frequencies, in two polarization planes. Radiance in the microwave region is usually expressed by the apparent brightness temperature $T_b = eT$, where T is the physical temperature of the emitting layer and e is its emissivity ($e \leq 1$). The emission comes from throughout the snow pack as well as from the underlying ground. The discrimination of surface types is possible mainly because of differences in emissivity. Figure 1 shows some examples. Whereas a snow-free soil shows little variation in emissivity over the whole frequency range, the emissivity of dry snow decreases with increasing frequency. This decrease depends on the thickness of the scattering layer and gives an indication of the water equivalent of the dry snow. The microwave penetration depth of wet snow is small owing to the high dielectric losses of water, so that the microwave signatures of dry and wet snow differ significantly. Regions undergoing a daily freeze-thaw cycle, commonly observed over melting snow fields in spring, can be recognized by comparing day and night microwave signals. However, a snow layer which is continually wet cannot be distinguished from snow-free ground by our algorithm, which may underestimate the snow fields undergoing heavy melt⁶.

The preceding snow cover parameters can best be obtained by an analysis of the 18 and 37 GHz horizontally polarized

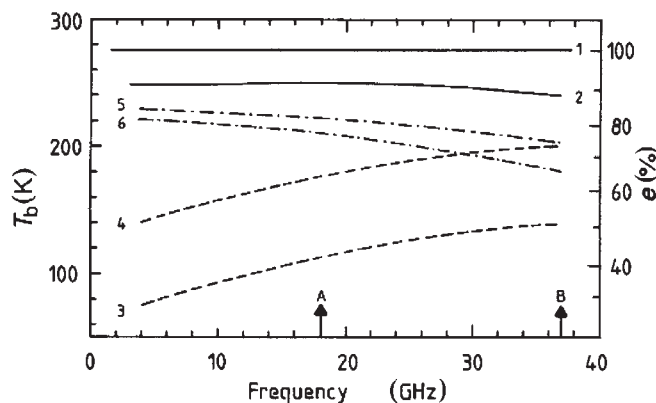


Fig. 1 Brightness temperatures (T_b) and emissivity (e) at microwave frequencies for: (1) an ideal black-body (assumed physical temperature of 273 K); (2) snow-free ground; (3) and (4) a flat sea surface, (3) horizontal and (4) vertical (physical temperature ~10 °C, incident angle ~50°); (5) snow-covered ground or old sea ice and (6) with increasing snow depth (horizontal). Curves (2), (5) and (6) are typical values obtained from ground-based microwave sensors¹⁵ and curves (3) and (4) are based on theoretical calculations and measurements¹⁶. A and B mark the SMMR frequencies used for snow retrieval.

SMMR channels⁷, with spatial resolution of 60×60 km and 30×30 km, respectively. The data from three days and nights, during a five-day interval, give complete coverage for areas polewards of 25° latitude. Because of satellite power limitations, data are recorded only on alternate days. The fully automated analysis consists of reading the data from tape, applying the retrieval algorithm and displaying the data on a colour screen. A pixel is classified as covered with dry snow when the difference in brightness temperature between the 18 and 37 GHz data is less than a prescribed threshold determined empirically from tests with ground data from throughout the middle and high latitudes of the Northern Hemisphere. Additional thresholds enable further differentiation between cover of more than 10 cm and thin or patchy snow. The result shows the maximum extent of snow cover over the five day period. The algorithm is described in more detail by Kunzi *et al.*⁷.

To assess the accuracy of the microwave analysis, we compared the SMMR data with snow charts based on shortwave imagery from March 1979. The central Asian region was selected because its terrain and vegetation are highly variable, because high-quality cloud-free imagery in the visible is common at this time of the year and because it is climatologically important⁸⁻¹⁰. As only a limited number of ground stations in this region report snow, only remote techniques can be used.

Snow was charted from the shortwave imagery collected daily by a polar-orbiting Defense Meteorological Satellite Program sensor with a spectral range of 0.4–1.1 μm and subtrack resolution of 2.8 km (ref. 11). Imagery was available on ungridded positive film transparencies on a scale of 1:15 million. A trained observer examined visually the transparencies from consecutive days and plotted the snow coverage on a gridded base-chart. Snow cover was differentiated from clouds and snow-free land by its characteristic surface brightness, textures and persistence^{12,13}. Two classes, full and partial cover, were distinguished. Areas where persistent clouds completely obscured the surface throughout the study period were charted as such and excluded from the analysis.

Microwave and shortwave charts for the intervals 11–15 March and 21–25 March 1979 were compared. No adjustments were made to improve the apparent agreement. Figure 2 shows the charts for 11–15 March. Grid points at the intersections of every 2.5° of longitude (50°E–140°E) and 1° of latitude (28°N–60°N) were categorized according to charted microwave and

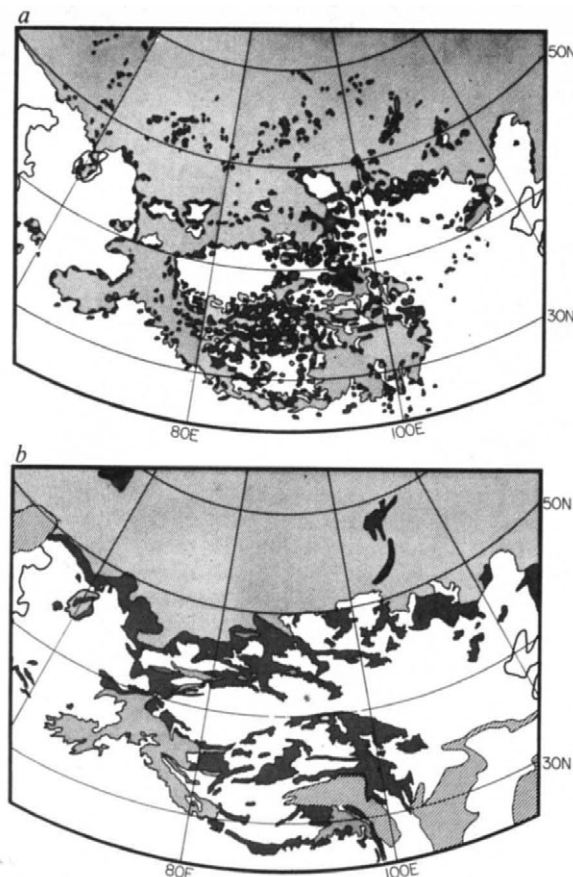


Fig. 2 Scanning Multichannel Microwave Radiometer (a) and Defense Meteorological Satellite Program (b) derived Asian snow cover for 11-15 March 1979. Fully snow-covered ground is shown in light stipple, partially covered is in dark stipple and snow-free is blank. Persistent cloudiness in b is hatched.

shortwave conditions. The results are shown in Table 1. Only 3.5% and 4.5% of the 924 gridpoints analysed on the 11-15 March and 21-25 March charts, respectively, were excluded because of persistent cloud cover. However, in other seasons clouds are more frequent in this region. Areas classified on the microwave charts as having dry snow deeper than 10 cm were assumed to be fully covered.

There is reasonable agreement between the microwave and shortwave classifications of surface conditions. Complete agreement is found over 76.4% (11-15 March) and 73.5% (21-25 March) of the area, whereas only 3.9% and 8.6% show full snow cover in one chart and no snow in the other (Table 1).

The best agreement between the charts occurs in areas where shortwave charts show full cover and where microwave charts show snow-free ground. Microwave charts tend to indicate more snow than the visual analysis. This is true for situations where partially covered areas in microwave analyses are classified as snow free in shortwave charts, and where full cover on microwave charts is seen as partial cover in the shortwave. Disagreement is most frequently observed over the Tibetan plateau, where snow-free ground intersperses the snow-capped mountains. Possible reasons for this are: (1) the much higher spatial resolution of the shortwave chart enables the recognition of individual snow patches covering only a small portion of an area, whereas microwave signals from these snow patches are combined with those of the adjacent snow-free ground and the area as a whole is classified as partially snow-covered; (2) imagery employed in this study may reflect time differences of up to 4 days, so that the snow observed in one set may have been absent in the other; (3) night-time dry-sand microwave

Table 1 Comparison of microwave and shortwave-based snow classification in per cent of the total number of gridpoints

11-15 March 1979

Shortwave classification	Microwave classification			Sum shortwave
	Fully snow-covered	Partially covered	Snow-free	
Fully snow-covered	39.7	2.5	0.5	42.7%
Partially covered	8.4	3.5	2.6	14.5%
Snow-free	3.4	6.2	33.2	42.8%
Sum microwave	51.5%	12.2%	36.3%	

21-25 March 1979

Shortwave classification	Microwave classification			Sum shortwave
	Fully snow-covered	Partially covered	Snow-free	
Fully snow-covered	35.3	2.8	2.4	40.5%
Partially covered	4.5	2.7	3.1	10.4%
Snow free	6.2	7.4	35.5	49.1%
Sum microwave	46.0%	12.9%	41.0%	

signatures are similar to those of thin dry-snow layers because the frequency-dependent penetration depth in dry sand is approximately several wavelengths and vertical temperature gradients in the sand on clear nights are strong.

The Scanning Multichannel Microwave Radiometer has now provided satellite data for almost six consecutive winter seasons in the Northern Hemisphere. Additional tests with shortwave satellite images and, where available, correlative ground data will further our understanding of the microwave data set. At present the multispectral microwave method appears to be very promising for automated large-scale charting of snow. This will prove invaluable, especially in autumn and early winter when snow fields develop in poorly illuminated areas under heavy cloud cover, making conventional charting difficult and inaccurate¹⁴. However, full substitution of shortwave snow-cover analysis by microwave mapping will not be possible in climate studies as one of the most critical snow parameters, its high albedo, cannot be assessed using microwaves.

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Surface faulting in the southern Italian Campania-Basilicata earthquake of 23 November 1980

Rob Westaway & James Jackson

Bullard Laboratories, Department of Earth Sciences,
University of Cambridge, Madingley Road,
Cambridge CB3 0EZ, UK

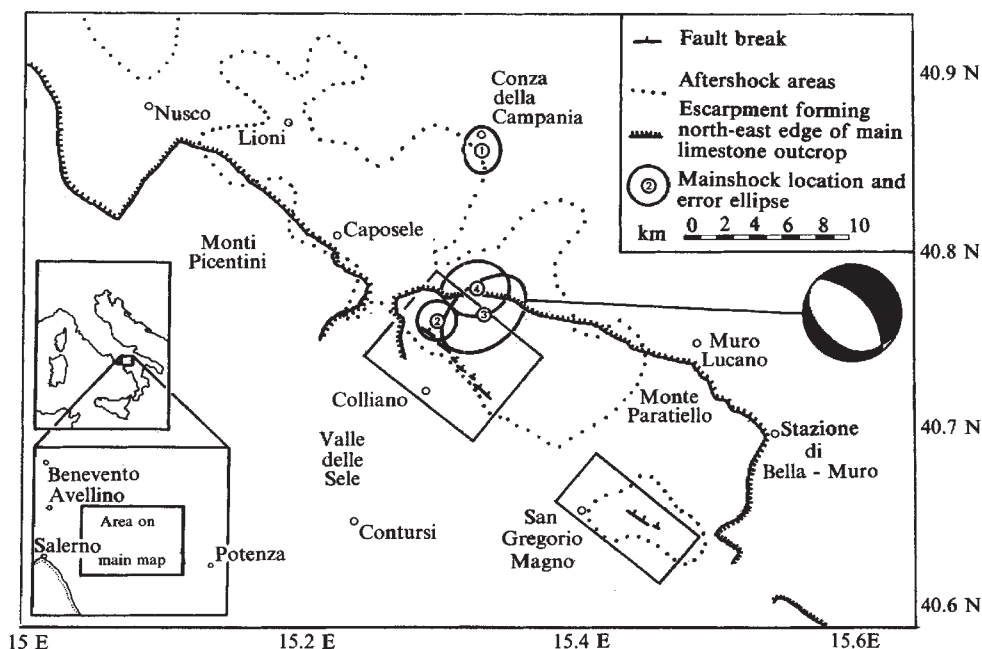
The Campania-Basilicata earthquake of 23 November 1980 was the largest ($M_s = 6.9$) and most destructive earthquake that has occurred in this region of southern Italy for over 100 years, killing more than 3,000 people. The consensus among seismologists, geologists and earthquake engineers who have studied¹⁻¹⁰ this event is, in keeping with previous Italian earthquakes, that it did not produce faulting at the surface. This has led to the establishment of a number of models^{2,3,11-13} requiring an unusual tectonic setting for the southern Apennines. However, on a recent visit to the epicentral region of the earthquake (May 1984), we discovered more than 10 km of surface faulting that is consistent with focal mechanisms for this event, based on first motion polarities^{2,4,5,12} and long period waveform studies¹⁴⁻¹⁶, which show that it involved normal faulting with one nodal plane dipping at $\sim 60^\circ$ towards the north-east. Our observations of this—the first well-documented earthquake fault break to be reported in Italy—suggest that the deformation associated with earthquakes in the southern Apennines takes place in the upper 10–15 km of the Earth's crust on steep planar normal faults which break the surface, as is widely observed in other areas of active extension outside Italy.

We found most of the fault break at altitudes of $> 1,300$ m in remote limestone mountains, covered with dense beech forest,

far from habitation or lines of communication. This area remained snow-covered until the spring following the earthquake and, consequently, the fault break escaped detection at the time of the earthquake. In the spring of 1981 the area was visited by many scientists (for example, the authors of refs 6–10, 17). Geologists from CNEN (Comitato Nazionale dell'Energia Nucleare) photographed parts of the break and, having interpreted it as a landslide, sent us their photographs, prompting this subsequent investigation. Our observations, when combined with the seismological evidence discussed below, strongly suggest that these surface breaks are of primary tectonic origin, and are not superficial effects.

Figure 1 shows the two separate segments of fault break identified. The longer segment is situated near the north-west end of the Monte Marzano–Monte Paratiello massif, to the east of the upper Sele valley (Figs 1, 2a), and consists of several strands each striking at approximately 300° and downthrown to the north-east. It extends for ~ 10 km from Piano Acquafredda, southeastwards to Piano Neurale. Between the strands it steps to the right, making its overall strike closer to 320° . The vertical displacement across the break is ~ 1 m in the middle of each strand, but smaller towards the ends. Much of this segment was well-known to local shepherds and labourers, who confirmed that it did not exist before the 1980 earthquake. Observations of a part < 2 km long, around the Piano di Pecore, have already been reported^{9,10}. These authors believed the break to be a secondary feature due to slipping or subsidence of superficial sediments. However, the individual strands of the break are straight, cutting across the topography. The overall trend of this segment is linear and crosses from the northeastern side of the high topography to the southwestern side near the col of Piano di Pecore. South-east of locality 5 in Fig. 2a, the uphill side of

Fig. 1 Positions of the observed fault break of the 1980 earthquake in relation to mainshock and aftershock locations. The focal mechanism for the mainshock (lower focal hemisphere, equal area projection, compressional quadrants shaded) is by Westaway and is based on first motion polarities observed at long period stations of the World-Wide Standard Seismograph Network. The north-east dipping nodal plane (dip 60° , strike 315°) is well constrained, and the other nodal plane (dip 30° , strike 125°) has been drawn to give the mechanism the same rake as in the observed fault break (80°), assuming the north-east dipping plane is the fault plane. This second nodal plane is not well constrained; its strike can be varied by up to 20° without introducing an inconsistency with any observed first motion polarities. Mainshock location 1, constrained at 0 km depth, is that of the ISC (International Seismological Centre), obtained from arrival times at 506 teleseismic stations. Location 2, at 16 ± 1 km depth, by del Pezzo *et al.*¹², was obtained using 11 Italian regional stations within 200 km of the epicentre. All the stations used lie in the north-west or south-east quadrants, and this inadequate azimuthal coverage makes the location of the epicentre potentially unreliable. Location 3, at 16 ± 2 km depth, was obtained by Westaway using the location program HYP071²⁵ with data from 49 regional stations within 400 km. Because Balkan and Sicilian stations are now included, the azimuthal coverage is much better and the horizontal coordinates are better determined, but as with location 2, the focal depth is not well determined, even though the nominal uncertainty in depth, calculated by the location program, is small in both cases. Location 4, at 15 ± 8 km depth, was obtained by Westaway by relocating the mainshock relative to a large aftershock²⁶, the location of which is well determined by the temporary local seismograph network. Sixty-three stations were used in this relative relocation. Depth is not well determined in any of the locations 1–4, although preliminary waveform modelling⁴ and aftershock locations suggest that the mainshock nucleated somewhere in the depth range of 10–15 km. Although our preferred location is 4, numbers 2, 3 and 4 are all in about the same place and are certainly expected to be more reliable than the ISC location. Although the reported errors for the ISC location are small, it is systematically misplaced to the north of the true location, a feature common to many teleseismic locations in the Mediterranean area, for which most of the stations recording any earthquake are to the north. The assumption that the ISC location is correct has led to the suggestion⁴ that the 1980 mainshock was caused by the motion of a fault whose surface expression is the large topographic escarpment extending south-east from Laviano past Muro Lucano as far as Bella-Muro railway station. This escarpment forms the northeastern edge of the Monte Marzano–Monte Paratiello limestone outcrop. The observations presented here do not support this suggestion.



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the rupture is downthrown. Along the break there are frequent exposures of a limestone fault surface with a consistent dip and strike, which at several places (for example, localities 1, 3 and 6 in Fig. 2a) appears to have moved in the past. The overall pattern of successive strands stepping to the right implies that the normal fault has a small component of left-lateral strike slip, and agrees with the sense of the motion at the one place where it could be directly measured (locality 7 in Fig. 2a). These observations strongly suggest that this segment is a fault break, not a landslide, and, as the largest aftershock was of only $m_b = 4.8$ ($M_s = 4.4$), the fault probably moved in the mainshock itself.

Figure 1 compares the position of this break with aftershock locations obtained from a network of more than 30 temporary seismographs installed in the epicentral area following the mainshock^{3,12,18,19}. In the centre of this network, aftershocks could be located with an accuracy of $\sim \pm 1$ km in horizontal coordinates and $\sim \pm 2$ km in depth. Most aftershocks are shallower than 12 km, with some as deep as 15 km. A clear feature of Fig. 1 is the cutoff in aftershock locations within 1 km of the position of the north-west segment of fault break. Very few aftershocks occurred south-west of this.

The second segment of fault break, with a throw of ~ 0.5 m, was found in the Pantano di San Gregorio about 5 km east of the village of San Gregorio Magno, situated about 16 km south-east of Piano di Pecore (Fig. 2b). Although only 3 km long, this

south-east segment has a similar strike to and lines up along strike with the north-west segment. A cluster of large aftershocks (Fig. 1) has been located under this segment, although, being situated near the edge of the temporary seismograph network, their locations are less reliable than those further north-west. This evidence and that of a limestone fault surface observed at locality 9 (Fig. 2b) suggest a primary tectonic origin for the surface displacements in this segment also. Moreover, as discussed below, the north-west segment cannot, by itself, account for all the faulting in the mainshock.

The displacements measured in the field are consistent with the observed drop in elevation obtained by levelling¹ in 1981 along a road running approximately parallel to but 8–12 km north-east of a line joining the two observed segments of fault break. Since the first levelling in 1959, the elevation of the road between Bella-Muro railway station (Fig. 1) and a point 6 km north of Laviano (Fig. 2a) had decreased by 20 cm in the east and up to 70 cm in the west, presumably as a result of the 1980 earthquake.

Figure 1 shows relevant seismological observations, including a focal mechanism and four possible epicentral locations for the mainshock, of which location 4 is probably the most reliable. A planar downward projection of the surface faulting (mean dip 62° north-east, strike 320°) passes beneath epicentre 4 at a depth of 10 km with an orientation almost the same as that of

Fig. 2 Detailed maps of the location: *a*, of the northwestern (Piano di Pecore) segment of fault break; *b*, of the southeastern (San Gregorio) segment. *a*, Between Piano Acquafredda and Piano di Pecore, the northwestern segment runs for 6 km along a northeast-facing escarpment, which rises to the summits of Monte Valva and Monte Marzano and which is mapped as a fault offsetting Cretaceous limestone. This escarpment continues 4 km further north-west to the edge of the limestone outcrop, and, though it is possible that faulting continued along this section also, it could not be traced because of the rugged topography, the thickness of scrub, and the debris from landslides covering much of this area. At locality 1, close to Piano Acquafredda, the break cuts a little-used forest track, and is visible in the track cutting. It is clear that at this point the same fault plane has moved in the past, as alluvium on the north-east side has been faulted down against limestone. The break can be followed through the forest to locality 2, where a 100-m long continuous limestone face (dip 63° , strike 304° , throw 1 m) with a smooth surface of cemented fault breccia is exposed. At locality 3, the break follows an abandoned forest track which appears to have been built along a ledge created by past motion of the same feature. At 4, one of the localities first noted in 1981^{9,10}, it descends the mountain side and cuts a road, originally forming a step with an offset of 1 m, now filled in, but still clearly visible. Close to the road the break resembles a landslide, but to the north-west it follows a uniform strike (310°) for 1 km, with frequent exposed planar surfaces of polished limestone breccia along it. To the south-east it is visible as a 0.5-m high scarp, downthrown to the north-east, and crossing Piano di Pecore; a small, flat, alluvium-filled basin on the northeast side of the escarpment. This basin is ponded by the escarpment and drains through it to the south-west. Further south-east, the break is again visible close to the top of the ridge separating Piano di Pecore from Piano Neurale. Along most of this section, the downthrown side, still to the north-east, is uphill. At locality 5, there is another exposure, 50 m long, of a planar limestone surface (dip 62° , strike 306° , throw 0.8 m) covered with fault breccia. At locality 6, the break occurs on the southwest side of a 100-m long gully, which a stream has eroded to a depth of 4 m into the limestone, separating it from the uphill slope to the north-east. This again suggests that the same feature has moved previously, before the 1980 earthquake. Further south-east, the break descends the south-west face of the ridge before finally dying out close to Piano Neurale. An extensive search further south-east failed to yield any evidence of continuation. At locality 7 (Figs 3, 4) the fault break occurred on a polished planar limestone surface with strike 298° and dip 60° . The base of this surface was stained by alluvium and indicates a throw of about 0.5 m during the earthquake. Striations on the stained surface indicate a slip vector with rake 80° north-west, that is, with a small (10°) component of left lateral motion. This is consistent with the right-stepping nature of the entire north-west segment, a feature observed in many other earthquakes, for example, ref. 27. *b*, Photographs¹⁷ of the southeastern segment in the Pantano di San Gregorio, taken in 1981, showed a surface break on this plain at locality 8 and, although the area has subsequently been ploughed over, the vertical offset of ~ 0.5 m, which strikes at 306° and is downthrown to the north-east, is still visible west of the road running around the south side of the plain. On the other side of this road, at locality 9, the rupture follows a polished limestone fault surface exposed at the edge of the plain (dip 68° , strike 298° , throw 0.4 m). This rupture could be traced for ~ 500 m before it became lost in the scrub. This segment may have extended further north-west of the Pantano di San Gregorio, but this could not be verified as the north-west edges of the plain, and the surrounding hillsides, have been covered with rubble dumped from the shattered village of San Gregorio Magno.

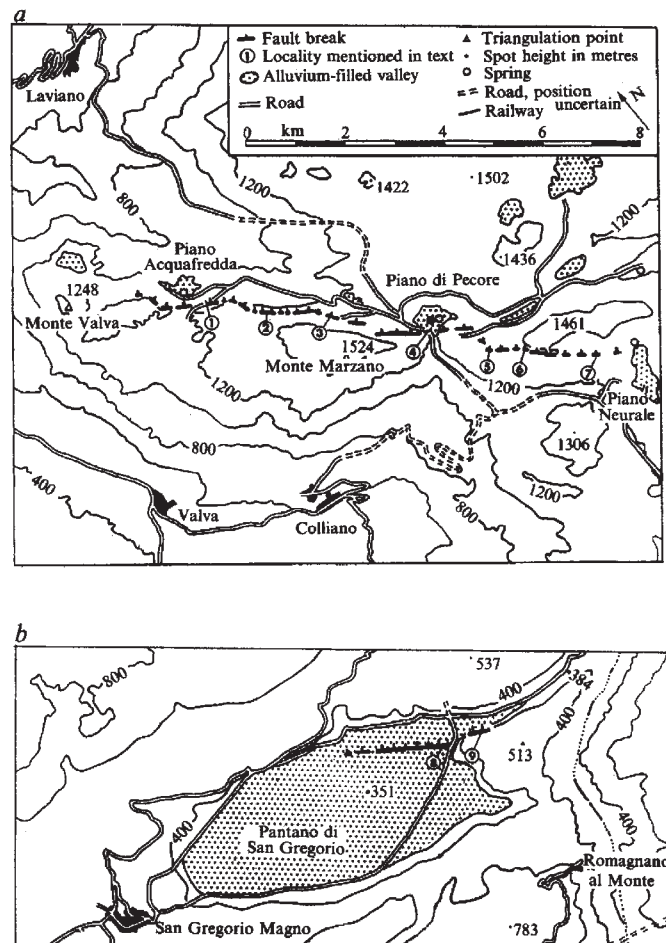




Fig. 3 The fault break looking north-west from locality 7. This is one of the few places where the surface break crosses open ground.



Fig. 4 Close up photograph, facing south-west, of the fresh striations (parallel to the pencil), stained by alluvium, observed on the fault at locality 7, indicating a small left-lateral component of strike slip.

the north-east dipping nodal plane in the focal mechanism of the mainshock (dip 60° north-east, strike 315°). Our observations are thus consistent with a planar fault geometry and a nucleation depth of 10 km for the mainshock. This depth is within the uncertainty in depth for location 4. Such a planar geometry is seen for other active normal faults whose source parameters are well resolved²⁰.

However, it is clear that the observed break on the north-west fault segment cannot account for all the motion that took place during the mainshock. The seismic moment of the mainshock, obtained by a moment-tensor inversion of long period body and surface waves¹⁴, is 3×10^{19} N m. Assuming a displacement of 1 m on a fault that extends 10 km horizontally, and from the surface to 15 km depth at a dip of 60° (corresponding to the north-west fault segment), the moment released would be only 6×10^{18} N m in rocks with a shear modulus of 3×10^{10} N m⁻². Additional faulting must have occurred elsewhere, and in the absence of any other information, the clusters of aftershocks shown in Fig. 1 may indicate further segments where faulting occurred during the mainshock. The ends of these segments occur at places where major structural discontinuities, such as that marked by the Sele valley²¹, intersect the structural and topographic trend. To account for the moment estimated from moment-tensor inversion, the amount of slip on each of these segments would need to be less than 1 m. This type of segmented fault geometry, controlled by structural discontinuities on a scale of ~ 10 km, appears to be common for large crustal earthquakes (see, for example, ref. 22).

The 1980 earthquake was the largest to have occurred in this region of southern Italy since the event of 16 December 1857, which occurred about 80 km further south-east and was studied by Mallet²³. He reported no surface faulting associated with the 1857 event, but he was in the epicentral area only between

February and March 1858, and was unable to examine the high mountains then covered in snow. Consequently, surface faulting in the 1857 event may have occurred, but remained undetected.

Between 1857 and 1980, two earthquakes comparable in size to the 1980 event occurred in the same area. Neither of these events on 23 July 1930 ($M_s = 6.5$)² and 21 August 1962 ($M_s = 6.2$)²⁴, was well recorded, and the uncertainty in the hypocentral location in both cases is much greater than for the 1980 event. The reported depths of 40 km (ref. 24) and 28 km (ref. 2) for the 1962 event are unreliable, particularly as the closest instrument to record this event unambiguously was more than 500 km distant. Thus, the 1980 event provides the main body of reliable information on which to base an understanding of the neotectonics of the southern Apennines; the evidence presented here suggests that deformation in this region occurs in the brittle part of the upper crust, on steep planar normal faults which break the surface.

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A Grenville Sm–Nd age for the Glenelg eclogite in north-west Scotland

I. S. Sanders*, P. W. C. van Calsteren†
& C. J. Hawkesworth†

* Department of Geology, Trinity College, Dublin, Ireland

† Department of Earth Sciences, The Open University,
Milton Keynes MK7 6AA, UK

Eclogite, a garnet–pyroxene rock with basaltic chemistry, is a product of unusually high-pressure metamorphism. In Scotland, it occurs within the Caledonian orogenic belt close to the Moine thrust, where it forms partially amphibolitized, easterly-dipping layers, associated with metasediments in the eastern Glenelg Lewisian inlier^{1–4}. In spite of its potential importance for an understanding of the geological history of north-west Scotland, its age has never been established more precisely than 'probably Proterozoic'⁴. A K–Ar omphacite age of 1,515 Myr⁵ is rendered unreliable by the possible presence of excess argon. We report here the results of an attempt to date the eclogite metamorphism by the Sm–Nd method. Two samples of eclogite yielded mineral isochrons of just over 1,000 Myr. The results represent clear evidence of Grenville metamorphism in Scotland, and imply close proximity of its front to the Moine thrust near Glenelg. Also, as the local Moine cover has not apparently reached eclogite facies, it was presumably deposited on an eroded Grenville basement.

The two samples selected for analysis, S215 and S274, are fresh, coarse-grained (>2 mm), granoblastic rocks: S215 consists of pale green, omphacitic pyroxene, pink garnet and some interstitial pargasite; S274 has more deeply coloured omphacite and garnet, and contains much quartz. Both rocks carry accessory rutile and show slight breakdown of omphacite to pyroxene–plagioclase symplectite. Mineral and rock compositions are given in Table 1: garnet and omphacite are unzoned; the rocks are meta-tholeiites. The eclogite samples were collected from the tract of country extending south-west from Loch Duich,

where the effects of ?Caledonian amphibolitization are minimal⁴. S215 comes from a thick eclogite layer overlying a spectacular cliff of marble, above Lochan na Beinn Fhada (grid ref. NG 860234); S274 comes from another thick layer at Cruachan Eilgeach (NG 841212).

The results of the analyses are presented in Table 1 and Fig. 1. The mineral–whole rock isochron ages, $1,010 \pm 13$ Myr and $1,082 \pm 24$ Myr ($\lambda^{147}\text{Sm} = 6.54 \times 10^{-12} \text{ yr}^{-1}$, 2σ uncertainty), are interpreted as reflecting the closure of Sm and Nd isotopic exchange between garnet and omphacite during post-metamorphic cooling. The blocking temperature for this system has been estimated as 600 °C (ref. 6); it was probably reached relatively soon after eclogite formation, as the high pressure of metamorphism (>12 kbar, see below) suggests that the crust was unusually thick, and that isostatic recovery would have been

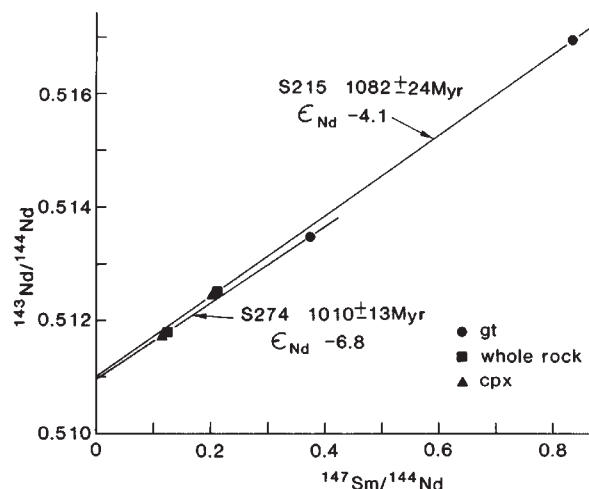


Fig. 1 Sm–Nd mineral whole-rock isochrons from two samples of Glenelg eclogite. ϵ_{Nd} is the initial $^{143}\text{Nd}/^{144}\text{Nd}$ ratio expressed as the deviation in parts per 10^4 from the contemporaneous Nd isotope ratio of the bulk earth.

Table 1 Major and rare earth element data for eclogite samples

	S215 rock	S215 gt	S215 cpx	S274 rock	S274 gt	S274 cpx	S135 rock
SiO ₂	47.33	39.02	53.76	49.33	38.57	53.56	52.46
TiO ₂	1.29	ND	0.28	3.31	0.05	0.28	1.59
Al ₂ O ₃	12.60	22.02	7.65	12.89	21.71	8.67	13.26
Fe ₂ O ₃	13.73	—	2.09	17.89	—	2.87	15.19
FeO	—	22.12	3.75	—	26.57	5.62	—
MnO	0.28	0.64	0.06	0.18	0.53	0.02	0.26
MgO	10.54	8.41	10.39	6.22	5.86	7.98	6.61
CaO	12.93	7.78	17.15	8.83	6.69	14.54	8.79
Na ₂ O	2.53	—	4.45	2.03	—	5.50	1.86
K ₂ O	0.04	—	0.01	0.04	—	—	0.07
Total	101.27	100.00	99.60	100.72	99.98	99.04	100.09
La	—	0.439	2.59	—	12.7	57.1	—
Ce	—	1.18	21.5	—	33.7	106	—
Nd	6.30	1.61	31.6	34.7	24.5	67.0	9.28
Sm	2.10	2.22	10.6	6.91	15.2	12.8	3.16
Eu	—	1.24	2.89	—	7.27	2.98	—
Gd	—	6.76	8.28	—	31.3	8.12	—
Dy	—	11.3	3.46	—	35.5	3.31	—
Er	—	7.01	0.884	—	18.5	1.17	—
Yb	—	6.33	0.467	—	15.0	0.826	—
$^{147}\text{Sm}/^{144}\text{Nd}$	0.2015	0.8348	0.2029	0.1206	0.3753	0.1156	0.2059
$^{143}\text{Nd}/^{144}\text{Nd}$	0.51243 ± 4	0.51696 ± 5	0.51247 ± 1	0.51179 ± 2	0.51348 ± 2	0.51175 ± 1	0.51221 ± 1

Clean garnet and clinopyroxene fractions in the size range +60–120 mesh were prepared using a Frantz-type magnetic separator. Mineral separates and rock powders were finely ground in a small agate ball mill, leached for 30 min in 6 M HCl and dissolved in a mixture of HF and HNO₃ in sealed Teflon bombs. Samples were taken up and aliquots collected for spiking in 2.5 M HCl. Rare earth element analysis was done in three fractions using standard isotope dilution techniques²⁰. Nd isotopes were measured as the metal species, normalized to $^{146}\text{Nd}/^{144}\text{Nd} = 0.7219$. Repeated analyses of BCR-1 yielded $^{143}\text{Nd}/^{144}\text{Nd} = 0.51262 \pm 2$. Rock analyses were by X-ray fluorescence spectroscopy²¹ on previously ignited samples. Mineral analyses were by Cambridge Geoscan²². Fe in garnet was taken as FeO. $\text{Fe}^{2+}/\text{Fe}^{3+}$ ratios in pyroxene were obtained by Mössbauer spectroscopy at Trinity College, Dublin. ND, not determined.

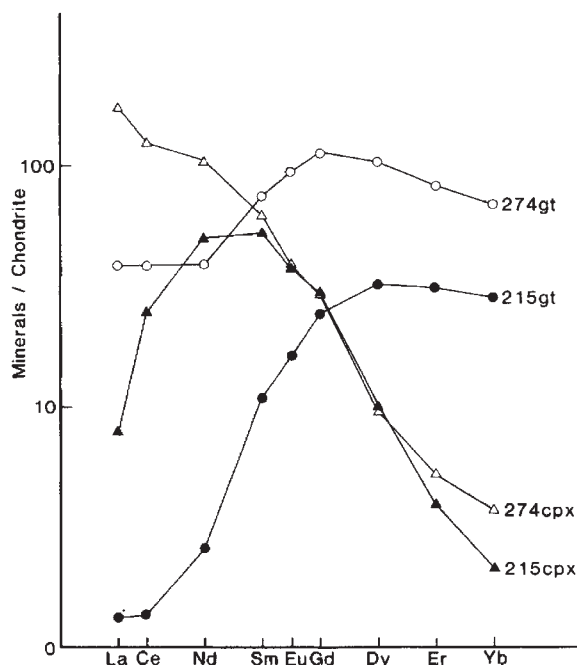


Fig. 2 Rare earth element variations in coexisting garnet-clinopyroxene pairs.

rapid. We infer, therefore, that metamorphism occurred at $\sim 1,100$ Myr, that is, during the Grenville orogeny. Of course, it is possible that the eclogite metamorphism was even earlier, and that Grenville overprinting caused thermal resetting of the isotopes without otherwise affecting the mineral compositions. However, this seems unlikely because retrogressive metamorphism observed at Glenelg is, at most, of lower amphibolite facies ($< 600^\circ\text{C}$) and Sm-Nd mineral isochrons are known to survive such metamorphic conditions⁷. The isochrons presented here have certainly survived Caledonian reworking.

Also, consistent with the estimated blocking temperature, equilibrium distribution of rare earth elements seems not to have occurred in eclogites formed below $\sim 600^\circ\text{C}$ (ref. 8). Where, as in the present case (Fig. 2), equilibrium was achieved, the distribution coefficient $K_D^{\text{gt-cpx}} (= \text{Sm}/\text{Nd}_{\text{gt}}/\text{Sm}/\text{Nd}_{\text{cpx}})$, where gt and cpx represent garnet and clinopyroxene respectively) will presumably vary inversely with temperature. The Glenelg eclogites ($K_D = 3.6\text{--}4.1$) therefore imply a higher closure temperature than the Scourie granulites ($K_D = 4.6\text{--}5.8$) even though the latter record higher metamorphic temperatures. The higher closure temperature at Glenelg is thought to reflect more rapid cooling.

A Grenville age for the eclogite is significant because it implies that the eastern Glenelg Lewisian lies east of the Grenville front. The age supports the suggestion that the basement to the Caledonian belt in Scotland underwent Grenville reworking⁹, and it leads us to speculate that the Moine thrust is a reactivation of the older Grenville front. The precise location of the Grenville front in the vicinity of Loch Duich appears to be the boundary between the eastern and western Glenelg Lewisian, as the latter preserves hornblende and biotite K-Ar ages of 1,600–2,200 Myr¹⁰.

What was the tectonic framework for generating eclogite so close to the Grenville front? The compositional data in Table 1 permit a general estimate of metamorphic temperatures and pressures to be made. Garnet-clinopyroxene geothermometry suggests a temperature close to 700°C (ref. 11), at which a pressure > 12 kbar would be required to stabilize eclogite of tholeiitic composition¹². Textures in associated eclogite facies feldspathic gneisses indicate intense, *syn*-metamorphic shearing on planes now dipping gently to the east⁴. The observed combination of pressure, temperature and strain suggests crustal imbrication, the Grenville crustal segment possibly having been thrust westward over the Hebridean craton.

What are the implications of Grenville eclogite in the basement for the depositional age of the unconformably overlying Moine metasediments? In marked contrast to the conditions for eclogite facies recorded in the basement, the Moine cover along the western margin of the Caledonian orogenic belt shows no sign of having exceeded conditions for lower amphibolite facies¹³. Also the local Moine stratigraphy straddles the boundary between the eastern and western Lewisian¹⁴. We therefore favour the view that Moine sediments were deposited on a deeply eroded Grenville basement. Viewed in this light, the Rb-Sr whole-rock isochrons of $\sim 1,000$ Myr for the southwestern Moines^{15–17} would be interpreted as dating post-tectonic sedimentation and diagenesis rather than regional metamorphism. The $^{87}\text{Sr}/^{86}\text{Sr}$ initial ratio, which is uniform at 0.709 over a wide area¹⁷, may reflect the average value for the source of the Moine sediments. Incidentally, the $\sim 1,000$ Myr isochron from the Torridonian Stoer Group has the same initial ratio¹⁸.

Returning to the eclogites, the Nd model age for the whole-rock sample with a low Sm/Nd ratio (S274) is $\sim 1,700$ Myr. This is the time at which the igneous precursor to the eclogite would have formed from mantle material of chondritic Sm/Nd and $^{143}\text{Nd}/^{144}\text{Nd}$ ratios¹⁹. The age is consistent with the basement east of the Moine thrust having experienced considerable mid-Proterozoic magmatism⁹. However, it would be unwise to attach too much significance to this model age as the other whole-rock sample analysed (S215), together with a third (S135 from grid ref. NG811164, Table 1), yielded unrealistic Nd model ages. Both have near-chondritic Sm/Nd ratios, but low $^{143}\text{Nd}/^{144}\text{Nd}$ ratios, which may reflect partial isotopic exchange with 'old' Nd in the associated Lewisian metasediments, possibly at the peak of eclogite metamorphism.

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Tocopherols as likely precursors of pristane in ancient sediments and crude oils

H. Goossens*, J. W. de Leeuw*, P. A. Schenck* & S. C. Brassell†

* Delft University of Technology, Department of Chemistry and Chemical Engineering, Organic Geochemistry Unit, de Vries van Heystplantsoen 2, 2628 RZ Delft, The Netherlands

† University of Bristol, School of Chemistry, Organic Geochemistry Unit, Cantock's Close, Bristol BS8 1TS, UK

Pristane and phytane, C_{14} and C_{20} isoprenoid alkanes respectively, are major components of many ancient sediments and crude oil. The phytol side chain of the chlorophyll *a* molecule was believed to have been the natural source of these hydrocarbons, but the mechanism by which phytane and pristane are generated from the phytol moiety of chlorophyll, under geological conditions, remain

unclear. Here we suggest that tocopherols, also known as E vitamins, are likely sources of pristane found in ancient sediments and crude oils, as both flash pyrolysis and thermal degradation of α -tocopherol yield prist-1-ene as a major product. The organic moieties in the kerogens from which prist-1-ene is generated were previously shown to be similar to those yielding pristane under natural conditions¹. As a consequence, pristane and phytane might very well originate from different precursors.

Fifty years ago, Alfred Treibs² demonstrated the presence of porphyrins in ancient sediments and crude oils. These compounds indicated the biological origin of petroleum: their common tetrapyrrolic skeleton suggested that they were derived from chlorophylls biosynthesised in photosynthetic organisms. Thus, the abundance of phytane and pristane found in extracts of ancient sediments and crude oils in the early days of gas chromatography^{3,4}, was quite reasonably thought to have come from the major chlorophylls *a* and *b*, which have a phytol side chain with an isoprenoid carbon skeleton similar to that of phytane and pristane.

The abundance of these isoprenoid hydrocarbons in geological materials has stimulated organic geochemists to investigate possible diagenetic pathways by which these compounds can be formed from phytol. Simulation experiments with phytol^{5,6}, and also *in situ* incubation experiments with ¹⁴C-phytol^{6,7}, have established the conversion of phytol to other functionalised isoprenoids in recent sediments. Unsaturated isoprenoid hydrocarbons such as phytene, phytadienes and phytol dimers and trimers are main products when phytol is heated to 60 °C under different conditions⁶. In thermodegradation experiments with intact sediments at relatively high temperatures (100–300 °C)^{8,9}, phytane, pristane and other isoprenoids are produced; these are all considered to be degradation products of the phytol side chain of the chlorophyll molecule. It is questionable, however, whether such thermodegradation studies accurately simulate natural diagenetic processes. During the past twenty years other isoprenoid compounds have been found in organisms and may also be considered as direct or ultimate sources of acyclic isoprenoid hydrocarbons in sediment samples—for example, phytylesters in aquatic mosses¹⁰, pristane and pristenes in zooplankton¹¹ and diphytanylethers in archaebacteria^{12,13}.

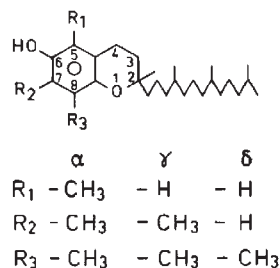


Fig. 1 Structure of tocopherols.

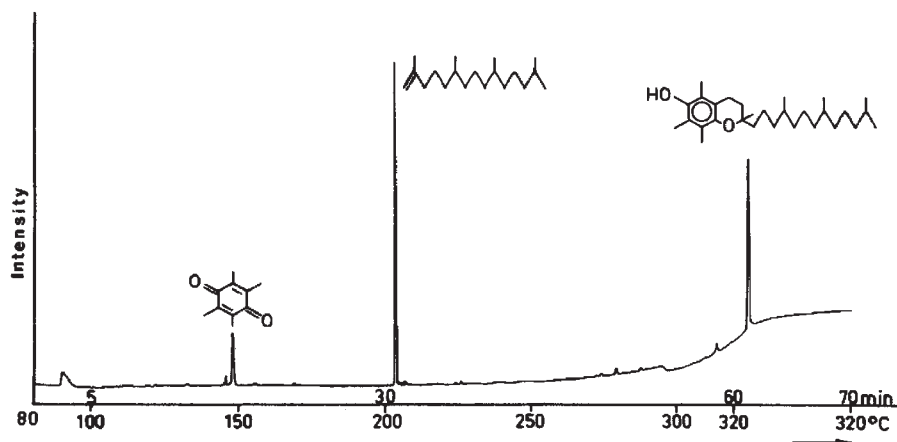
Flash pyrolysis studies of many immature sediments and kerogens have demonstrated that prist-1-ene is a major and sometimes dominant product in the pyrolysate^{14,15}. The flash-pyrolysates of certain organisms and the uppermost layers of sediments have also been found to contain small amounts of prist-1-ene^{14,16–18}.

A detailed study of a suite of Paris Basin samples of increasing maturity showed a good correlation between maturity and the concomitant disappearance of the pyrolysis product prist-1-ene and the generation of pristane¹. This phenomenon indicates that a common precursor, present in the kerogen, generates pristane during maturation, and prist-1-ene on flash pyrolysis. The ability of several isoprenoids to generate prist-1-ene on flash pyrolysis has been assessed to find possible biological precursors for pristane. Dihydrophytyl-palmitate¹⁸, chlorophyll *a*¹⁸, diphytanylethers, phytol and dihydrophytol (J. W. de Leeuw *et al.*, unpublished results) were found to yield mainly phytene or phytadienes. Prist-1-ene is either not present or is only a trace component in the pyrolysates, except for that from palmitylphytanate¹⁸. Although esterified phytanic acid is present in very recent sediments^{19,20}, it is either absent or present in trace amounts in sediments beyond the early stages of diagenesis. Thus, the isoprenoid compounds investigated by flash pyrolysis are not considered to be major sources of pristane in ancient sediments and crude oils. In addition, the type of structure which generates prist-1-ene on flash pyrolysis differs from that which yields straight chain hydrocarbons as these occur as a series of alk-1-enes and alkanes, whereas pristane is absent even when prist-1-ene is the most abundant component in the pyrolysates¹⁴.

Recently, tocopherols (see Fig. 1) have been found in a wide variety of sediment extracts^{21,22}. Their common occurrence in sediments suggests that these compounds—either free or bound—may have an important role in the genesis of isoprenoid hydrocarbons. Therefore, α -tocopherol was pyrolysed under conditions routinely used to investigate kerogens by Curie-point pyrolysis–gas chromatography–mass spectrometry¹⁴. Two major pyrolysis products were encountered: 2,3,5,6-tetramethyl-2,5-cyclohexadiene-1,4-dione (duroquinone) and prist-1-ene (see Fig. 2). The compounds were identified by comparison of their retention times and mass spectra with those of reference compounds.

A thermal degradation experiment with α -tocopherol was also undertaken (N₂; 350 °C; 6 h) and the results were compared with those obtained by Fernholz in 1937²³. Our results support those of Fernholz to some extent: prist-1-ene and duroquinone were major products here, whereas the other components present such as C₁₃, C₁₆ and C₁₈-isoprenoid alkanes, were probably secondary products of prist-1-ene. Figure 3 shows the mechanism proposed for both the flash pyrolysis and thermal degradation. Because tocopherols are highly reactive compounds²⁴, which can easily react with each other and with other naturally occurring compounds, it is thought that the majority of tocopherols will be incorporated into a complex macromolecular

Fig. 2 Pyrolysis gas chromatogram of α -tocopherol. Conditions: Curie point pyrolysis at 610 °C; gas chromatographic separation on a fused silica column (length = 25 m; ϕ = 0.31 mm) coated with CPSil 5 (Chrompack); temperature programme: 80–320 °C at 4 °C min⁻¹, held at 320 °C for 15 min; flame ionization detector (FID) detection).



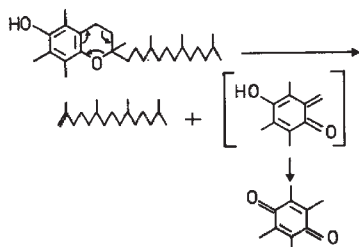


Fig. 3 Proposed degradation mechanism of α -tocopherol.

structure, probably via the phenolic hydroxyl group or the 5-methyl group which is very reactive under oxidative conditions. However, their major pyrolysis product should nevertheless be prist-1-ene formed by the intramolecular rearrangement indicated in Fig. 3. The stereochemistry of pristane in immature sediments²⁵ is also consistent with that of α -tocopherol²⁶.

The flash pyrolysis results obtained for α -tocopherol and the presence of free tocopherols in many sediments^{23,24} suggest that tocopheryl moieties can be major sources of pristane in ancient sediments and crude oils. Tocopherols are relatively abundant in most photosynthetic organisms such as higher plants, algae and cyanobacteria²⁷⁻³¹. Recently, many bacteria have been reported to contain tocopherol derivatives³². Amounts of 10 to >1000-mg kg⁻¹ dry body weight are not exceptional for the concentration of free tocopherols in organisms. These quantities might be underestimates as almost nothing²⁹ is known about the occurrence of non-extractable tocopheryl moieties in biological systems.

The relatively great abundance of chlorophylls in photosynthetic organisms (molar ratio tocopherol/chlorophyll = 0.01–0.12²⁷) does not imply that other compounds, such as tocopherols, can be ruled out as precursors of pristane in the geosphere; the potential survival of a compound during early diagenesis may be more important. Early in diagenesis, phytol is liberated from the tetrapyrrole macrocycle of chlorophylls and is a labile compound. A very high percentage of the phytol biosynthesised by primary producers is probably metabolised within the food web. In tocopherols, by contrast, the isoprenoid moiety is bonded via a carbon–carbon bond to an aromatic nucleus, favouring survival during early (bio)chemical diagenesis. Moreover, tocopherols may escape biodegradation once incorporated into macromolecular structures.

In conclusion, although tocopherols can now be considered as likely precursors for pristane, in view of their pyrolytic behaviour, their relatively high concentrations in organisms and their expected survival by incorporation into kerogen, the generation of phytane from this source is less likely because α -cleavage next to the aromatic moiety is highly unfavourable. As a consequence, pristane and phytane may very well originate wholly or in part from different precursors.

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Accelerator mass spectrometry radiocarbon ages of amino acid extracts from Californian palaeoindian skeletons

J. L. Bada*, R. Gillespie†, J. A. J. Gowlett† & R. E. M. Hedges†

* Amino Acid Dating Laboratory (A-012B), Scripps Institution of Oceanography, University of California at San Diego, La Jolla, California 92093, USA

† Oxford Radiocarbon Accelerator Unit, Research Laboratory for Archaeology and the History of Art, Oxford University, 6 Keble Road, Oxford OX1 3QJ, UK

A decade ago, aspartic acid racemization ages were determined for some skeletal remains found in California, near La Jolla, Del Mar and Sunnyvale, suggesting that people were present in North America during the Upper Pleistocene^{1,2}. These ages were obtained from the aspartic acid racemization rate, which was calibrated using a radiocarbon date of $17,150 \pm 1,470$ yr BP determined for a skeleton found in Laguna Beach, California^{3,4}. These studies generated an intense controversy not only about the antiquity of human beings in the New World^{5,6} but also about the validity of racemization-based ages^{6–9}, and prompted efforts to date the finds by other means^{6,7,10,11}. Here we have used accelerator mass spectrometry (AMS) to determine the radiocarbon ages of the amino acid extracts used in the original racemization studies. Our studies indicate that some of the controversial Californian skeletons, which had been assigned to the Upper Pleistocene, are in fact Holocene.

The dating of the first human migrations into the Americas is one of the most controversial topics in prehistory. An accurate time scale for colonization is necessary to assess how rapidly hunters and gatherers could expand into virgin territory, and also to evaluate how much time was required for the development of the ethnic and linguistic diversity exhibited by the New World populations. Many archaeologists^{12,13} favour a 'late' entry of $\sim 12,000$ – $13,000$ yr BP. However, dates of this magnitude are found even for southern South America^{14–16}. Therefore it seems necessary to postulate that human groups first crossed Beringia much earlier, despite recent reviews of the archaeological evidence which find few convincing sites^{17,18}. Of central import-

Table 1 Radiocarbon and uranium series ages, extent of aspartic acid racemization and level of amino acid preservation in several palaeoindian skeletons from California

Label no.	Sample	Radiocarbon age Oxford	Other	U-series age	D/L-Asp	% Amino acids remaining	Ref.
OxA-152	Stanford	4,830 ± 150	5,130 ± 70		0.14	39	2
OxA-153		4,950 ± 130					
OxA-154	SDM 16709	8,470 ± 140	8,360 ± 75	3,350 ± 800	0.19	4–11	29, 31, 32
OxA-189	Laguna	5,100 ± 500	17,150 ± 1,470 > 14,800		0.25	5	1, 3, 4
OxA-186	La Jolla	5,600 ± 400	1,770 ± 790 1,850 ± 200 1,930 ± 200 5,331 ± 245 6,326 ± 253		0.36		1, 7
OxA-187	Sunnyvale	6,300 ± 400	3,600 ± 600 4,390 ± 150 4,650 ± 400 4,850 ± 400	8,900	0.51	1.4	2, 6, 11
OxA-188	Del Mar	5,400 ± 120		11,800	0.53	2	1, 2, 11

ance in this controversy are the ages of various palaeoindian skeletons found in western North America.

The total amino acids present in the various bone samples (5–10 g) were isolated using procedures described elsewhere^{19,20}. Enantiomeric analyses indicated that the aspartic acid obtained from the La Jolla Shores, Del Mar and Sunnyvale skeletons was extensively racemized. When compared with the extent of aspartic acid racemization (that is, D/L-Asp ratio) measured in the skeleton found near Laguna Beach, which had a conventional radiocarbon date of 17,150 ± 1,470 yr BP, these skeletons appeared to be 30,000–50,000 yr-old^{1,2}. A portion of the original amino acid extracts used in these racemization studies was preserved either frozen or as the dried residue. These extracts, containing 5–10 mg amino acids, together with those isolated from several other skeletons previously dated by radiocarbon, were prepared and analysed by AMS^{16,21,22}. Our AMS-radiocarbon ages and the measured D/L-Asp ratios are listed in Table 1, together with the radiocarbon and uranium-series ages obtained by others. The amount of amino acids preserved in the various bone samples (relative to that in modern bones) is also given in Table 1. The AMS-radiocarbon dates indicate that all of the skeletons, including Laguna, are of Holocene age.

The radiocarbon ages determined using the total amino acid extracts isolated from the Stanford (I) and SDM 16709 skeletons agree well with the ages determined from conventional radiocarbon measurements using the HCl-insoluble ('collagen') component. Our ages for the Sunnyvale skeleton are ~2,000 yr older than the radiocarbon ages of other organic fractions isolated from this skeleton, but the results still indicate that it is Holocene. Therefore we find that the ages obtained by analysis of the total amino acids are comparable with those obtained using either the HCl-soluble or the HCl-insoluble organic fractions.

The radiocarbon age obtained for the La Jolla Shores skeleton agrees with a previously determined AMS age¹⁰ of this skeleton, whereas those obtained using the conventional radiocarbon method are much younger²³. This may reflect the presence of bones of various ages on the site, or we must assume that the fractions used in the conventional measurements were extensively contaminated. In contrast, the AMS-radiocarbon age of amino acids isolated from the Laguna Beach Skeleton is much younger than that obtained, almost 15 years ago, by conventional beta-counting using the HCl-insoluble, gelatinized fraction^{3,4}. The difference in ages for this skeleton is difficult to account for, and may indicate that the total amino acids isolated were not entirely derived from collagen but also contained secondary amino acids from the surroundings. However, based on the good agreement between our amino-acid based ages and those obtained using other organic components, for several of the other skeletons studied, we conclude that Laguna is more likely to be Holocene than Upper Pleistocene as previously assigned.

If indeed the case, this implies that all the aspartic acid racemization ages derived for other specimens, using an age of 17,150 ± 1,470 yr BP for the Laguna skeleton, are too old. Using the newly-determined 5,100 ± 500 yr age for Laguna yields an aspartic acid racemization rate constant, $k_{asp} = 3.6 \pm 0.4 \times 10^{-5} \text{ yr}^{-1}$, giving ages for Sunnyvale and Del Mar of ~14,000 yr BP. Although this age still is older than the AMS-radiocarbon ages of the skeletons' amino acids, it does demonstrate that both Sunnyvale and Del Mar are not of very great antiquity. The k_{asp} value obtained using the 5,100 yr-old age for Laguna also suggests that the age of Los Angeles Man, previously estimated by the conventional radiocarbon method⁴ to be >23,600 yr BP, is more probably ~8,000 yr-old; thus the Upper Pleistocene age assignment for this skeleton should also be viewed with scepticism.

The results in Table 1 reveal no clear relationship between the radiocarbon ages of the various skeletons and the extent of aspartic acid racemization. For example, the 8,400 yr-old skeleton, SDM 16709, has a low D/L-Asp ratio, whereas in the Del Mar and Sunnyvale skeletons the aspartic acid is much more extensively racemized even though these skeletons are 2,000–3,000 yr younger. These results contrast with those obtained at other sites around the world, where in general the extent of aspartic acid racemization has been found to be directly correlated with the age of fossil bones^{19,20,24,25}.

Besides this lack of correlation there are also great differences in the amounts of amino acids preserved in the bones of the Californian skeletons of similar age. In fact, the samples in which aspartic acid is extensively racemized have the lowest amino acid contents and, indeed, there appears to be a direct relationship between the extent of racemization and the level of preservation of collagen (as judged by the amino acid contents of the bones). Bone collagen undergoes hydrolysis via a series of reactions, beginning with cleavage at labile peptide bonds such as those containing aspartyl and seryl residues^{20,26}. The peptides liberated by this hydrolysis are further degraded via peptide bond hydrolysis and an internal aminolysis reaction which takes place at the N-terminal end of the peptides^{20,27}. If, during the process of collagen hydrolysis, the aspartyl residues end up primarily at the N-terminal positions of the peptide hydrolysis products, this could give rise to rapid racemization rates as amino acids at the N-terminal position in peptides have been found to have racemization rates greatly exceeding those at other positions²⁸.

The skeletons having low amino acid contents were found to be depleted in 4-hydroxyproline and enriched in the acidic amino acids, although the general amino acid compositions of these samples still somewhat resembled that of collagen. This indicates that the collagenous matter in these bones had undergone severe diagenesis, which may have altered the D/L-Asp

ratio²⁹. The factors which have contributed to the extensive collagen hydrolysis in some of the skeletons are not known, but probably include leaching by ground waters and heating effects resulting from either shallow burial depths or cultural burial practices. Also many of these Californian skeletons come from coastal shell middens in which the presence of carbonates in the burial soils may have greatly accelerated the rate of collagen hydrolysis^{30,31}.

The results in Table 1 show that there are also difficulties with the application of the uranium (U-) series method for dating these Californian palaeoindian remains. In the case of SDM 16709, the U-series age is too young, whereas for the Del Mar and Sunnyvale skeletons the corresponding ages are too old. The U-series age appears to depend on the level of amino acids preserved in the bones. The basic assumption in U-series dating^{11,31} is that bones rapidly acquire uranium from the surrounding soils. Initially, this uranium is in the water-soluble U⁶⁺ oxidation state, but the bone organic matter acts to reduce and fix this to water-insoluble U⁴⁺, which then decays to the daughter isotopes Th and Pa. In this model, the U-series age of a bone will tend to be younger than the bone's actual burial age³¹, which is the case for SDM 16709³². Our results suggest that, for bones in which the organic matter has undergone severe decomposition, some of the uranium may be reoxidized and lost from the system resulting in U-series ages which are older than the actual burial age of the bone.

In conclusion, our results indicate that the palaeoindian skeletons from California, originally thought to be Upper Pleistocene, are more probably all of Holocene age. Moreover, the direct radiocarbon dating, by AMS, of the amino acids isolated from bones provides a means for determining accurate racemization rate constants which are essential in the amino acid racemization method for dating bones.

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Aequorin measurements of free calcium in single heart cells

P. H. Cobbold & P. K. Bourne

Department of Zoology, University of Liverpool, PO Box 147, Liverpool L69 3BX, UK

The performance of the heart depends on the concentrations of free calcium ions in the cytoplasm of the myocytes. However, direct evidence for changes in free Ca concentration in physiological events during response to drugs and in pathogenesis has been difficult to obtain because of technical problems in measuring free Ca at 10^{-7} M in cells with a volume of only a few picolitres¹⁻³. Here we describe measurements made with the Ca-sensitive photoprotein aequorin³⁻⁵ in single ventricular myocytes isolated from rat heart⁶. We have detected signals from resting and contracting cells, and from cells exposed to media of altered ionic composition (raised K, lowered Na), ouabain and metabolic inhibitors. We report that free Ca in metabolically-poisoned myocytes is remarkably stable and that severe injury to the cell occurs before the free Ca concentration rises above $1-3 \times 10^{-7}$ M, hence cell damage seems to be a cause, not a consequence, of a rise in free Ca. The technique used here should help to resolve many uncertainties regarding free Ca in heart function, and should be particularly valuable for investigating the role of free Ca in ischaemic pathogenesis.

Ventricle myocytes were isolated by perfusing rat hearts with collagenase⁶ and suspended in medium containing 1.8 mM Ca. Healthy, rod-shaped cells were injected to ~5% of their volume with aequorin solution from a micropipette having a tip external diameter of <0.5 μ m. Photons emitted from aequorin in a single myocyte were detected by a sensitive, low-noise photomultiplier (see ref. 5 and Fig. 1 legend for details). Figure 1a shows a resting signal from a single rod-shaped myocyte; the free Ca concentration in this cell was 2.5×10^{-7} M. The mean resting free Ca concentration for 42 cells was 1.8 ± 0.61 ($\pm$ s.d.) $\times 10^{-7}$ M, comparable with microelectrode measurements in Purkinje cells from several species³ and close to aequorin measurements made in ferret papillary muscle⁷. However, our value is probably an overestimate because of the contribution made to the signal by occasional phasic contractions in some of the cells in the sample. Similarly, it is possible that published resting free Ca measurements have been distorted by these small transients, so we are reluctant to place any significance on differences between published values for resting free Ca concentration. Figure 1b was recorded from a cell that showed phasic contractions (in which a localized contractile zone ripples from one end of the cell to the other in ~1 s) at a frequency of ~10 min⁻¹. When exposed to 10 μ M adrenalin, some myocytes started beating either regularly (~1 s⁻¹) or in bursts (Fig. 1c); these patterns are similar to published patterns of action potentials⁸. The peak height (~230 counts s⁻¹) and duration of the regularly-occurring transients (data not shown) were distorted by the slow response time of the recording apparatus, but if their true time course was as described by Allen and Orchard⁹, the number of counts in each transient, which was recorded faithfully by fast scaler, would correspond to a peak free Ca concentration of $\sim 3 \times 10^{-6}$ M, comparable with Allen's value. Some of the transients showed 'hesitations' on their downward slopes; improvements in data acquisition and analysis are needed to reveal these 'after-contractions' and, perhaps, oscillatory phenomena¹⁰. Figure 1c shows that each burst of transients (~2 s⁻¹) followed a 10-s period of elevated free Ca ($\sim 9 \times 10^{-7}$ M) which seemed to be unaccompanied by a change in membrane potential⁸. Slow action potentials, mediated by a very slow calcium current ($i_{Ca,s}$) have recently been described in guinea-pig myocytes¹¹. Spontaneous slow free Ca transients from a rat myocyte are shown in Fig. 1d. The peak free Ca concentration was 2.5×10^{-6} M, comparable with estimates for fast transients. Spontaneous slow

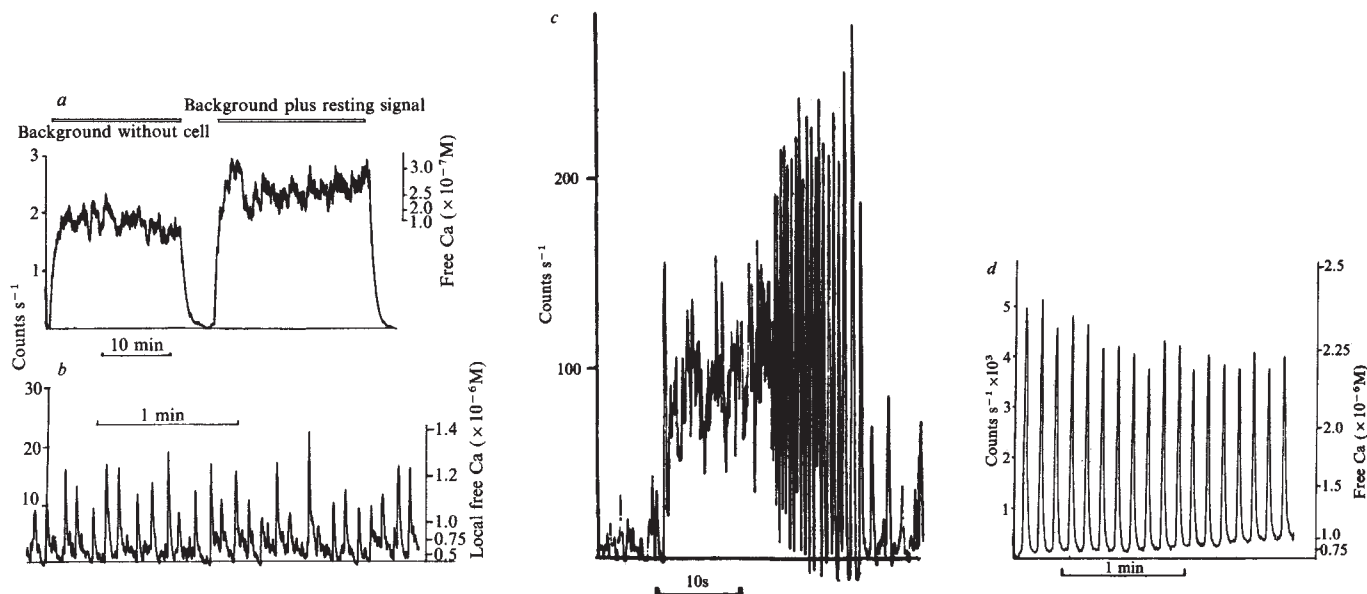


Fig. 1 Aequorin signals from single rat ventricle myocytes, recorded as photoelectron counts s^{-1} . **a**, Resting (diastolic) signal, 0.6 counts s^{-1} above background (1.8 counts s^{-1}). Ratemeter time constant 33 s. Free Ca concentration was 2.5×10^{-7} M, calculated from the rate of aequorin consumption⁵ knowing the total aequorin counts were 1.8×10^5 . (This recording is from the same experiment as Fig. 2b and corresponds to the first 20 min; note the rod-shaped appearance of the healthy cell.) **b**, Aequorin transients recorded from phasic contractions (ratemeter time constant 1 s). Peak height was ~ 12 counts s^{-1} , total aequorin content 9.4×10^5 counts, hence if the zone of contraction occupied 5% of the cell's volume, the local free Ca concentration would be $\sim 1.2 \times 10^{-6}$ M (see right-hand ordinate). Phasic transients were enhanced in amplitude and frequency in elevated extracellular Ca (3.8, 8 mM), abolished in low calcium medium (0.2 mM), but not detectably changed by either verapamil (10 μ M) or Ni (2 mM). **c**, A burst of spontaneous, fast contraction transients recorded from a myocyte exposed to 10 μ M adrenalin for 3 min before the start of the recording. The peak height and duration of the transients will have been distorted by the relatively slow ratemeter time constant (0.1 s) and by the mechanical inertia of the chart recorder (also responsible for the undershoot below 0 count s^{-1}). The cell contained 1.18×10^6 aequorin counts. **d**, Slow aequorin transients. Peak signals of $\sim 4 \times 10^3$ counts s^{-1} were recorded faithfully (0.33 s time constant) and corresponded to 2.5×10^{-6} M free Ca (2.57×10^6 counts remained in the cell). Diastolic signals of ~ 200 counts s^{-1} represented 8×10^{-7} M free Ca. These slow transients started spontaneously 5 min before the start of recording, following a 10-min period when the free Ca concentration rose from 3.5 to 9×10^{-7} M. The amplitude of the transients declined steadily for 6 min after the end of this recording and became unresolvable when diastolic free Ca was 3.2×10^{-6} M. From this criterion, the cell was judged to be in a poor condition.

Methods: The technique was modified from ref. 5 as follows. Aliquots (0.2 μ l) of concentrated aequorin solution (~ 30 mg ml^{-1}) were dialysed for 5 h in a microdialysis tubule against several 100- μ l changes of injection buffer containing (mM): 140 KCl (Aristar) 1 PIPES, 0.025 EGTA, 0.1 EDTA, pH 7.2. Borosilicate micropipettes were filled through the tip by means of a pressure chamber working on the same principle as the air-pressurized equipment described previously but filled with liquid paraffin (mineral oil). Oil pressure of up to 200 atm from an air-to-oil intensifier pump allowed a column of aequorin solution to be forced ~ 0.2 mm into the pipette tip. Myocytes suspended in agarose gel (2%; Sigma IX) within 0.1 mm of the open end of a 3 mm-long flat glass capillary (Camlab microslide) were then impaled with piezoelectric assistance under $\times 600$ magnification and injected with aequorin expelled from the micropipette under 0.3–4 atm air pressure. About 25% of injections were successful, the cells remaining rod-shaped; mean total aequorin content was 6×10^5 counts. Experiments were carried out in HEPES-buffered medium of normal calcium concentration (mM): 120 NaCl, 5.8 KCl, 4.3 $NaHCO_3$, 1.4 KH_2PO_4 , 1.5 $MgSO_4$, 1.8 $CaCl_2$, 18.7 HEPES, 14 glucose, pH 7.3 at 36–37 $^{\circ}C$.

transients have been found only in deteriorating cells with elevated diastolic free Ca (8×10^{-7} M in this cell). It is interesting that both the slow transients (Fig. 1d) and the bursts of fast transients (Fig. 1c) started when the free Ca concentration had risen to 8 – 9×10^{-7} M; the relationship between free Ca and the initiation of spontaneous transients requires further investigation in view of its possible importance in arrhythmias.

The involvement of free Ca in pathological changes brought about by metabolic inhibition, especially during ischaemia and reperfusion, has long been debated^{12,13}. Recent aequorin measurements in papillary muscle have shown that free Ca does not rise rapidly following anoxia or metabolic inhibition⁹; single myocytes responded similarly to inhibition of both oxidative phosphorylation and glycolysis. Figure 2a shows a typical result: exposure of a myocyte to cyanide (2 mM) together with 2-deoxyglucose (10 mM) had no effect on resting free Ca (2.5×10^{-7} M) for ~ 16 min, at which time the free Ca concentration rose slowly over a further 20 min to 5×10^{-6} M. Similar responses were found with the mitochondrial uncoupler CCP (carbonyl-cyanide phenylhydrazide, 0.5 μ M; Fig. 2b). In this experiment, recording of the signal was interrupted to view the cell under a microscope; shortening of the cell started 6 min after adding CCP and was complete 6 min later, yet the free Ca concentration remained at 3×10^{-7} M. In such experiments it was usual for

free Ca to remain at normal resting levels (~ 1 – 3×10^{-7} M), whereas the myocytes shortened to about one-third of their original length. Low concentrations of ATP (~ 10 – 100 μ M) cause tension development or shortening in permeabilized myocytes in zero free Ca^{14–16}; it is conceivable that a similar mechanism caused shortening of our poisoned cells, assisted by changes in the affinity of troponin-C for calcium at low ATP levels¹⁷. A contribution to shortening could arise from potentiation of tropomyosin-modulated myosin S1-ATPase at Mg-ATP concentrations of ~ 30 μ M¹⁸. On the other hand, Piper *et al.*¹⁹ measured levels of 500–1,000 μ M ATP in anoxic, predominantly rod-shaped myocytes that had shortened, on average, by 40%; this level of ATP appears to be at least 10 times too high for shortening to be explained by any of the above mechanisms.

Blockade of either oxidative phosphorylation or glycolysis alone usually had no effect on free Ca concentration or cell length. Cells that had rounded-up spontaneously in normal medium often showed normal, stable, resting free Ca concentrations. The slow rise in free Ca in rounded-up poisoned myocytes (Fig. 2a, b) occurred a few minutes after the appearance on the cell surface of large hyaline blebs ~ 10 – 30 μ m across, similar to those seen on myocytes after prolonged anoxia^{19–21}, whereas rounded-up cells with stable resting free Ca levels were devoid of these large blebs²².

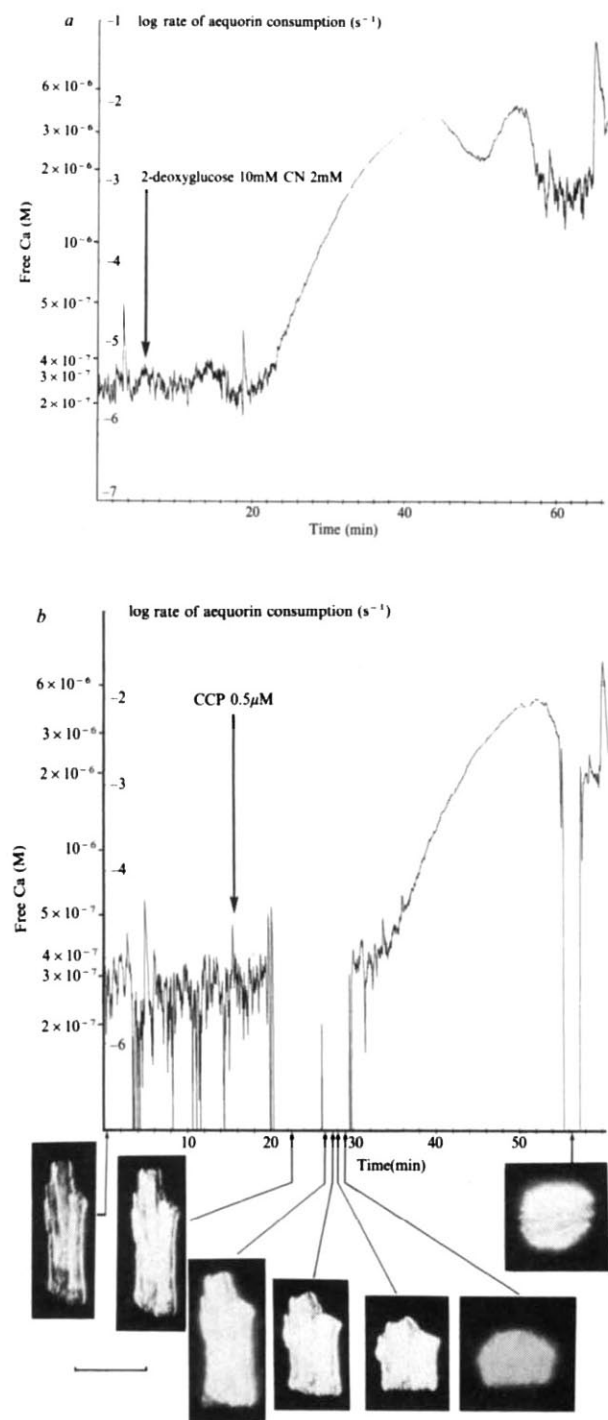


Fig. 2 Effect of metabolic inhibitors on free Ca in single myocytes. A microcomputer-based photon counting system was used to collect data and to plot the log rate of aequorin consumption (s⁻¹) at 1-s intervals. Values of free Ca derived from *in vitro* calibration curves⁵ are also plotted on the ordinate. *a*, Effect of CN (2 mM, fresh) together with 2-deoxyglucose (10 mM, in glucose-free medium). The free Ca concentration remained unchanged at 2.5×10^{-7} M for 16 min then rose over 22 min to 4×10^{-6} M. This is a typical result; the delay between addition of inhibitors and the onset of the rise in free Ca varied from 5 to 45 min. (The plateau at $\sim 5 \times 10^{-6}$ M may be an artefact caused by 'slow-reacting' aequorin; the reappearance of noise in the trace was due to the fall in the count rate as aequorin was consumed, resulting in a rise in statistical noise.) *b*, Effect of the mitochondrial uncoupler CCP (0.5 μM). The signal recording was interrupted to take photomicrographs of the cell, which show the time course of rounding-up of the cell. The free Ca concentration remained stable during this period at 3×10^{-7} M (confirmed by uninterrupted recordings of other cells, as in *a*). Scale bar, 50 μm.

We believe that the technique outlined here will help to resolve the relationship between free Ca, metabolic inhibition, altered excitability and structural injury in heart cells. From the preliminary evidence presented here, we conclude that the control of free Ca is not precariously dependent on an abundant supply of metabolic energy and is more resistant to perturbation than is maintenance of normal cell structure.

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Glucose induces closure of single potassium channels in isolated rat pancreatic β-cells

Frances M. Ashcroft, Donna E. Harrison* & Stephen J. H. Ashcroft*

University Laboratory of Physiology, Parks Road, Oxford OX1 3PT, UK

* Nuffield Department of Clinical Biochemistry, John Radcliffe Hospital, Headington, Oxford OX3 9DU, UK

The major physiological stimulus for the secretion of insulin from the pancreatic β-cell is an increase in the plasma glucose concentration. It is well established that glucose-stimulated insulin secretion is associated with the appearance of electrical activity in the β-cell^{1,2}; glucose concentrations above the threshold level for insulin release produce a slow membrane depolarization followed by either oscillatory bursts of action potentials (5–15 mM glucose) or continuous spiking (>16 mM glucose). Tracer flux studies³ and microelectrode measurements using intact islets of Langerhans⁴ have indicated that the initial depolarization induced by glucose is caused by a decrease in the resting membrane permeability to potassium. Evidence also suggests that the electrical⁵, ionic⁶ and secretory responses^{7,8} to glucose are mediated by the metabolism of the sugar within the β-cell. By using cell-attached membrane patches⁹ from isolated rat pancreatic β-cells, we have now identified a potassium channel (G-channel) that is active at the resting potential and is inhibited by glucose. Closure of this channel requires glucose metabolism. This is the first report of a potassium channel whose activity is modulated by glucose, and which may couple metabolic and ionic events involved in the secretion of insulin.

The β-cells were obtained from rat islets of Langerhans (see Table 1) and maintained in short-term tissue culture. For single-

channel recording, cells were allowed to attach to glass coverslips overnight; over 95% of attached cells stained positively for insulin using an immunoperoxidase method and anti-insulin antibody¹⁰, indicating that most were β -cells. The insulin content of the dispersed cells, determined by radioimmunoassay¹¹, was 3.7 mU per 10^4 cells, comparable to that reported previously¹². We also tested the secretory response of the isolated β -cells by measuring insulin release in response to glucose stimulation (Table 1). Insulin release was increased 2.4-fold by increasing the glucose concentration from 2 mM to 20 mM, and reduced to the basal level in the presence of mannoheptulose (20 mM), which inhibits β -cell metabolism of glucose at the level of hexose phosphorylation^{7,8}. The magnitude of the response to glucose is comparable to that reported previously for dispersed islet cells¹².

To investigate the effects of glucose on the resting membrane permeability to potassium, we recorded single-channel currents from cell-attached patches⁹ using patch pipettes filled with a solution containing 140 mM potassium. In glucose-free solution, the channel observed most frequently at the resting potential had a unit amplitude of approximately -3.5 pA (Fig. 1a). The single-channel current decreased as the membrane was depolarized and reversed at a pipette potential (V_p) of -74.8 ± 1.0 mV ($n = 16$). Figure 1b gives the current-voltage relation of the G-channel, which shows inward rectification. The single-channel conductance, measured between a pipette potential of +80 and -60 mV, had a mean value of 50.6 ± 0.9 pS ($n = 16$). Over the same potential range, the opening probability of the channel showed no significant voltage-dependence. Figure 1a also shows that the G-channel has complex kinetics, with episodes of long closures lasting for several hundreds of milliseconds and episodes of frequent rapid closures of only a few tens of milliseconds. This suggests that the channel has at least two closed states.

Using the whole-cell recording configuration⁹, we determined the β -cell resting potential to be -67.6 ± 2.8 mV ($n = 8$) in glucose-free solution, in agreement with microelectrode studies on intact islets^{2,4}. This value would indicate reversal of the single-channel current at a membrane potential of +7.2 mV, which is close to the estimated K-equilibrium potential of +8.5 mV (taking the internal K concentration to be 100 mM; ref. 4) and suggests that the G-channel is principally permeable to potassium. To confirm this, we carried out three experiments with 70 mM potassium in the pipette (NaCl replacing KCl). In these conditions the extrapolated reversal potential was -58.7 ± 1.3 mV ($n = 3$), corresponding to a 16-mV shift for a 70-mM

Table 1 Insulin secretion by dispersed islet cells

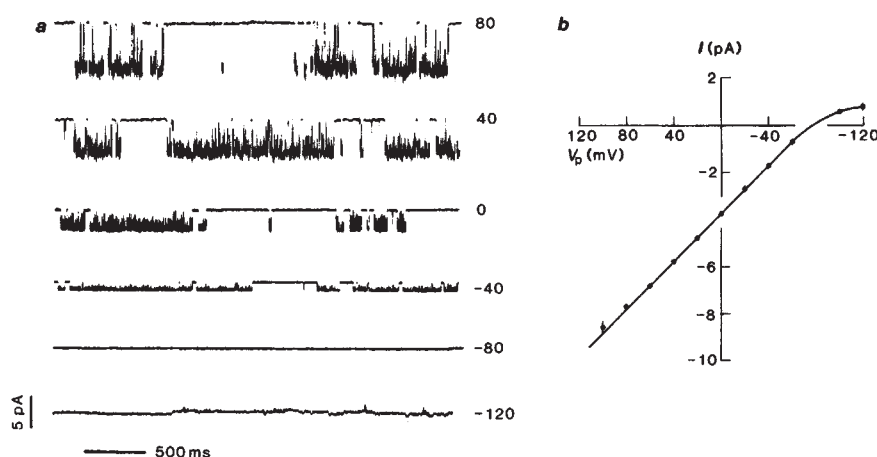
Incubation condition	Insulin release (μ U per 10^4 cells per h)
2 mM glucose	259 $\pm$ 21
10 mM glucose	384 $\pm$ 25 ($P < 0.01$)
20 mM glucose	623 $\pm$ 38 ($P < 0.001$)
20 mM glucose + 20 mM mannoheptulose	268 $\pm$ 19 (NS)

Isolated rat islets of Langerhans were prepared by collagenase digestion¹⁴ and dissociated into single cells by further enzymatic digestion and mechanical dispersion¹⁵. Single cells (2×10^4 cells ml⁻¹) were maintained in RPMI 1640 tissue culture medium supplemented with glucose (11 mM), penicillin (100 U ml⁻¹), streptomycin (0.1 mg ml⁻¹) and fetal calf serum (10%), and incubated at 37 °C in a humidified atmosphere of 95% air/5% CO₂. After overnight culture, cells were collected by centrifugation and washed twice in incubation medium. Incubations (10^4 cells per tube) were then carried out at 37 °C in 0.5 ml HEPES-buffered bicarbonate medium¹⁶ containing 2 mg ml⁻¹ bovine serum albumin and the additions stated. After incubation for 1 h, cells were removed by centrifugation and insulin content of the medium was determined by radioimmunoassay¹¹. Values are mean $\pm$ s.e.m. ($n = 10$). Significance of the differences (P), relative to 2 mM glucose, was assessed by a two-tailed Student's t -test. NS, not significant.

change in external K concentration, close to that predicted by the Nernst equation (14 mV) for a K-selective channel. The single-channel conductance decreased to 36.5 ± 0.9 pS ($n = 3$) with 70 mM K⁺ in the pipette.

The G-channel was found in 34 out of 41 membrane patches at an average frequency of 1.4 channels per patch. For our pipettes, which had resistances between 3 and 5 G Ω , the patch area is $\sim 5 \mu\text{m}^2$ (ref. 13), indicating a density of ~ 1 channel per $3.5 \mu\text{m}^2$ or roughly 150 channels per cell. We also identified two other channels in cell-attached patches, using pipettes filled with 140 mM K⁺ solution. One of these channels had a conductance of 16.9 ± 1.1 pS ($n = 10$) and a unit amplitude of -1.2 ± 0.1 pA ($n = 10$) at the resting potential of the cell, and was observed less frequently than the G-channel (11 out of 41 patches). The other channel was usually activated only at pipette potentials positive to +100 mV, had a unit amplitude of $\sim +6$ pA at +120 mV, a conductance of 119.6 ± 7.8 pS ($n = 8$) and an extrapolated reversal potential (pipette potential) of -74.4 ± 2.3 mV ($n = 8$). The frequency, conductance and voltage-dependence of these two channels suggest that they do not contribute a significant amount of conductance at the resting potential:

Fig. 1 a, Single-channel currents recorded from a cell-attached patch on an isolated rat pancreatic β -cell in glucose-free solution. The potential applied to the inside of the patch pipette (pipette potential) is given to the right of each trace; 0 mV corresponds to the resting potential of the cell (~ -70 mV) and positive pipette potentials correspond to membrane hyperpolarization. Inward currents across the membrane are shown as downward deflections of the current trace and channel openings are inward when the pipette potential is positive to -80 mV and are outward at -120 mV. Experiments were carried out on single cells (10–15 μm in diameter) prepared as described in Table 1, within 1–6 days after plating onto glass coverslips. Similar results were obtained when antibiotics were omitted from the culture medium. The patch pipette was filled with the following solution (in mM): 140 KCl, 5 CaCl₂, 5 MgCl₂ and 5 HEPES titrated to pH 7.4 with NaOH. The bath solution contained (in mM): 135 NaCl, 5 KCl, 5 CaCl₂, 5 MgCl₂ and 5 HEPES titrated to pH 7.4 with NaOH. The patch pipettes and recording system were similar to those described by Hamill *et al.*⁹. All experiments were carried out at room temperature (21–25 °C). No corrections have been made for liquid junction potentials¹⁷. Seal resistance = 25 G Ω . Current records were filtered at 0.2 kHz. **b**, Mean relationship between the single-channel current (I) and the pipette potential (V_p) ($n = 16$). Bath and pipette solutions were as described for **a**. The reversal potential corresponds to a pipette potential of -76 mV, and a pipette potential of 0 mV corresponds to the resting potential of the cell. The conductance, measured from the slope of the line between +60 mV and -60 mV, was 51 pS. The line through the points was drawn by eye. Vertical lines indicate $\pm$ s.e.m. where this is larger than the symbols.



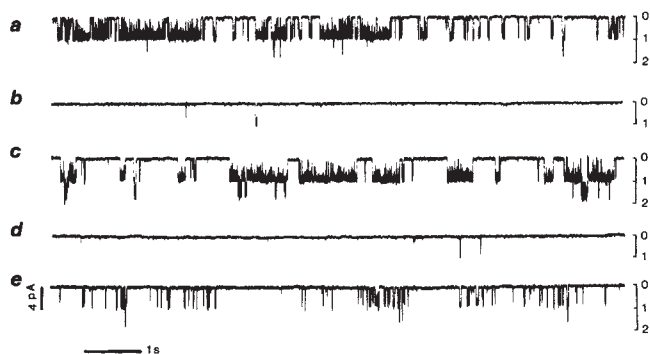


Fig. 2 Effect of glucose on G-channel activity. Single-channel currents recorded at a pipette potential of 0 mV: *a*, in glucose-free solution; *b*, 7 min after changing to a solution containing 20 mM glucose; *c*, 12 min after return to glucose-free solution; *d*, 4 min after perfusion with 20 mM glucose; *e*, 2 min after perfusion with a solution containing 20 mM glucose plus 20 mM mannoheptulose. Records were obtained successively from the same cell. This patch contained two channels and the scale on the right indicates the current level when one or both channels were open. After recovery of activity at the end of the experiment, we confirmed that we were in the cell-attached recording configuration by applying further suction and obtaining the whole-cell configuration⁹. The bath had a volume of 1 ml and was perfused at a rate of ~ 0.8 ml min⁻¹. A slow rate of perfusion was used to avoid rupture of the seal. Analysis of the perfusate indicated that the glucose concentration stabilized within 5 min. Temperature 25 °C. Currents filtered at 0.2 kHz. Seal resistance 25 G Ω .

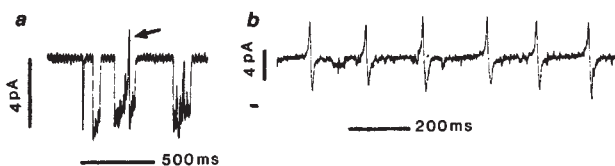


Fig. 3 *a*, Action potential (arrowed) elicited by the opening of a single G-channel in 10 mM glucose solution (12 min). Current waveforms associated with action potentials in the rest of the cell membrane may be recorded extracellularly from cell-attached membrane patches when the input resistance of the cell is high¹⁷. Note that the single-channel currents also decay in amplitude, indicative of a high input resistance. *b*, Example of spontaneous action potentials elicited in response to 20 mM glucose, recorded extracellularly from a cell-attached patch ~ 2 min after changing to glucose solution. In 20 mM glucose, extracellularly recorded action potentials usually decreased in amplitude and become difficult to measure with time. Records filtered at 0.2 kHz.

rather, our results indicate that the resting potential is principally determined by the G-channel.

Figure 2 shows the modulatory action of glucose on the G-channel. Application of 20 mM glucose to the bath resulted in the rapid and reversible inhibition of G-channel activity. In all of six experiments, 20 mM glucose produced a substantial

reduction in channel activity which reached a steady state within 3–10 min and in four of these experiments, glucose removal restored activity over a similar time course. We were unable to test reversal in the other patches because of spontaneous rupture of the seal. Glucose completely abolished G-channel activity in four cases. We were unable to determine the latency of inactivation of the single-channel currents very accurately because a slow perfusion rate was required to avoid rupture of the seal. In the presence of 10 mM glucose, G-channel activity was also substantially reduced (five out of seven patches), three of these showing almost complete inhibition.

Inhibition of channel activity was accompanied by a transient increase in the input resistance of the cell and by the appearance of action potentials. The increase in input resistance was shown both by the relaxation of single-channel currents and by the ability of a single-channel opening to trigger an action potential (Fig. 3*a*). Continuous spiking usually occurred in the presence of 20 mM glucose (Fig. 3*b*), as reported previously for intact islets^{2,4,5}. It is unlikely that the disappearance of the G-channel in the presence of glucose results simply from membrane depolarization because the opening probability of the G-channel is not significantly voltage-dependent between +80 and -60 mV (V_p). Further, channel activity was not restored in the presence of glucose when the pipette potential was changed by 120 mV in either direction.

It is well established that glucose-induced electrical activity⁵, reduced K permeability⁶ and insulin secretion^{7,8} all require the metabolism of glucose. Therefore, in three experiments, we tested the effect of inhibiting glucose metabolism with 20 mM mannoheptulose. In all cases, perfusion with 20 mM glucose plus 20 mM mannoheptulose restored channel activity previously abolished by glucose (Fig. 3*e*). Insulin release from the same preparation of single cells was also inhibited by this concentration of mannoheptulose (Table 1). This suggests that the effect of glucose on channel activity is a consequence of its metabolism.

Thus, we have shown that glucose can inhibit the activity of a channel which contributes significantly to the resting potassium permeability of the β -cell. As the G-channel is normally open at rest, its closure can account for the decrease in membrane potassium permeability^{3,4} and consequent depolarization^{1,2} produced by glucose. G-channel closure must result from an intracellular action of glucose because the pipette solution did not contain glucose and the patch membrane was isolated from the sugar in the bath by the high resistance of the glass-membrane seal. Although we cannot exclude the possibility that glucose and mannoheptulose compete directly for a common receptor site on the G-channel, there is considerable evidence^{6,7} that the effects of glucose and mannoheptulose on both resting potassium permeability and insulin secretion have a metabolic basis. Our results are fully consistent with the hypothesis that channel modulation results from an interaction between some metabolic product of glucose and the G-channel protein(s).

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T-cell control of Epstein-Barr virus-infected B cells is lost during *P. falciparum* malaria

Hilton C. Whittle, Jim Brown, Kevin Marsh,
Brian M. Greenwood, Peter Seidelin,
Helen Tighe & Lucy Wedderburn

Medical Research Council Laboratories, Fajara, near Banjul,
The Gambia

Endemic Burkitt's lymphoma, a tumour of children in which B lymphocytes are infected with Epstein-Barr virus (EBV), is common in areas of Africa where malaria is holoendemic¹. The tumour is characterized by chromosome translocations²; usually the terminal portion of chromosome 8 containing the *c-myc* gene is translocated to chromosome 14³, near the enhancer of the immunoglobulin heavy-chain locus⁴. Less frequent are translocations of chromosome 8 to the κ light-chain locus of chromosome 2 or to the λ light-chain locus of chromosome 22⁵. *In vitro*, EBV induces B cells to proliferate and secrete immunoglobulin and antibody⁶. However, *in vivo* the infected B lymphocytes are under immunological control, so that abnormal proliferation is found only in immunosuppressed patients^{7,8}. Such patients are subsequently liable to develop lymphomas^{7,9}. Burkitt believed that the tumour he had described resulted from interaction between a virus(es) and a "reticuloendothelial system altered by chronic and heavy infection by malarial or other parasites"¹⁰. We report here that during an attack of *Plasmodium falciparum* malaria, T-cell subpopulations are radically altered so that, *in vitro*, B lymphocytes infected with EBV proliferate abnormally to secrete large amounts of immunoglobulin and antibody. This phenomenon offers some explanation for the increased incidence of Burkitt's tumour and the high levels of immunoglobulin found in people living in areas where *P. falciparum* malaria is common¹¹.

Venous blood was taken from patients aged 5-18 yr (mean 9.8 ± 4.7 yr) during and 3 weeks after an acute attack of *P. falciparum* malaria. Parasite densities ranged between 0.5% and 8% (mean $3.1 \pm 2.7\%$). All patients had antibody titres of at least 1 in 20 to the viral capsid antigen of EBV. Mononuclear cells were separated by buoyant gradient centrifugation on Lymphoprep and used in the regression assay¹² or for determination of lymphocyte subpopulations. Regression assays provide a measure of the strength of T-cell control of EBV-infected B lymphocytes¹². We found that regression indices were high (mean 3.3 ± 1.2) during the attack, denoting loss of control, but that they fell on recovery (mean 1.0 ± 0.4 ; $P < 0.001$, paired *t*-test), signifying a return to normal control (Fig. 1). These 'escaped' B cells produced large amounts of immunoglobulin and a variety of antibodies (Table 1).

It was possible that the autologous plasma in which the regression assays were performed contained a B-cell mitogen¹³ or activator¹⁴, causing proliferation and secretion of immunoglobulin by EBV-infected B cells. To test this idea, we assayed

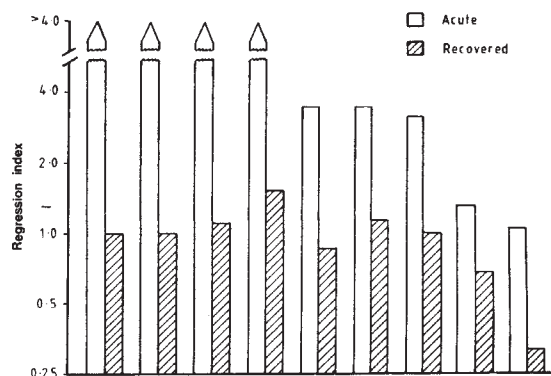


Fig. 1 Regression assays of nine children during the acute and recovered phase of *P. falciparum* infection. The regression index is the minimum number of cells $\times 10^5$ per 0.2 ml microtitre well which are necessary for a 50% frequency of regression of EBV-infected lymphocytes. Mononuclear cells were separated from venous blood on Lymphoprep, infected with EBV at a multiplicity of infection of 0.1 by exposure to B95-8 supernatant for 1 h, washed and then suspended in RPMI-1640/20 mM HCO_3^- with 15% autologous plasma. Doubling dilutions of cells starting at 4×10^5 per 0.2 ml and ending at 0.25×10^5 per 0.2 ml were placed in each of six wells of a flat microtitre plate, incubated for 4 weeks at 37 °C in 5% CO_2 in air. Half the medium was changed weekly and the cultures were examined for the presence of transformed B cells, characterized by proliferating colonies with hair-like projections, or for regression of these colonies, characterized by dense packing of colonies and then disintegration¹². The 50% frequency of regression was calculated by the method of Reed and Muench²⁵. For statistical purposes, an index of >4.0 was assigned an arbitrary value of 4.1. The mean index of eight healthy adult controls, immune to EBV, was 1.14 ± 0.86 .

mononuclear cells of two normal EBV-immune adult donors in media containing 10-15% of the patient's acute- or recovered-phase plasma. The mean regression indices of six paired assays for the first donor were 0.42 ± 0.14 in acute-phase plasma and 0.66 ± 0.16 in recovered plasma; for the second donor, using five different pairs of plasma, the respective means were 2.12 ± 1.25 and 3.27 ± 1.16 . When the assays were performed in fetal calf serum, the regression indices were 0.40 for the first donor and 2.00 for the second donor.

Having confirmed that plasma factors were not responsible for the high regression indices found during the malarial attack, we explored the possibility that T- and B-cell numbers had been altered by the infection. Table 2 shows that during the attack the number of T cells was reduced; this included both the number and percentage of T-helper cells (T_H) and the ratio of helper to suppressor cells (T_H/T_S). The proportion of B cells was increased, as was the ratio of B cells to suppressor T cells (B/T_S). These findings may explain the high regression indices, for the index is dependent on the number and power of cytotoxic suppressor T cells in the assay¹². These cells were unlikely to have been fully functional, because suppressor cell activity is

Table 1 Immunoglobulin and antibody production by transformed B lymphocytes in regression assays

Patient	Regression index	Immunoglobulin ($\mu\text{g ml}^{-1}$)		Antibody (end dilution)		
		G	M	<i>P. falciparum</i> IgG	EBV VCA	Sheep red cells
1 Acute	4.0	>25.0	58.0	>100	10	16
1 Recovered	1.1	3.2	46.0	10	0	<4
2 Acute	4.0	>25.0	50.0	$>1,000$	1	32
2 Recovered	1.5	3.2	34.0	10	0	<4

Regression assays were performed as described previously¹² except that media contained 15% fetal calf serum (FCS) instead of plasma. After 4 weeks, supernatants from all wells were pooled and tested. IgG and IgM was measured by an enzyme-linked immunosorbent assay²³, *P. falciparum* antibody by an immunofluorescent method using acetone-fixed parasitized red cells as targets, antibody to EBV viral capsid antigen (VCA) by immunofluorescence using the method of Henle *et al.*²⁴ and antibodies to sheep red cells by direct haemagglutination.

Table 2 Number ($\times 10^3$ per μ l) and percentage of T and B cells in blood of 14 patients with *P. falciparum* malaria

Stage of infection	Total lymphocytes		T lymphocytes		T _H lymphocytes		T _S lymphocytes		B lymphocytes			
	No.	%	No.	%	No.	%	No.	%	T _H /T _S	No.	%	B/T _S
Acute	1.57 $\pm$ 0.83	19.2 $\pm$ 7.5	1.00 $\pm$ 0.66	62.6 $\pm$ 13.0	0.53 $\pm$ 0.38	31.9 $\pm$ 7.2	0.46 $\pm$ 0.26	30.6 $\pm$ 9.3	1.1 $\pm$ 0.3	0.40 $\pm$ 0.27	24.7 $\pm$ 8.7	0.9 $\pm$ 0.4
	2.92 $\pm$ 1.09	65.0 $\pm$ 12.5	1.99 $\pm$ 0.93	67.5 $\pm$ 10.7	1.47 $\pm$ 0.72	50.8 $\pm$ 7.3	0.57 $\pm$ 0.25	20.3 $\pm$ 4.9	2.6 $\pm$ 0.7	0.32 $\pm$ 0.21	10.6 $\pm$ 3.4	0.6 $\pm$ 0.3
P value	<0.001	<0.001	<0.001	NS	<0.001	<0.001	NS	<0.001	<0.001	NS	<0.001	<0.05

Mononuclear cells were separated on Lymphoprep, washed, suspended at 3×10^6 ml⁻¹ in RPMI/20 mM HEPES/20 mM HCO₃ containing 15% FCS, placed in 1.5-ml aliquots in 2-cm² wells of a tissue culture plate (Linbro) and incubated overnight at 37 °C. In the morning, all non-adherent cells were removed by pipetting, washed in cold phosphate-buffered saline (PBS)/1.0% FCS/0.1% azide and aliquots reacted at 4 °C with the following antibodies: fluorescein-conjugated F(ab')₂ fragment rabbit anti-human immunoglobulin (Cappel) and OKT3, OKT4, OKT8 mouse monoclonal antibodies (Ortho). Cell-bound mouse monoclonal antibodies were detected by staining the cells, after washing, with fluorescein-conjugated rabbit anti-mouse IgG (Miles Yeda) which had been preabsorbed with human IgG bound to formalin-fixed *Staphylococcus aureus* cells. A drop of each stained lymphocyte suspension was spread on a glass slide, fan dried, fixed in ethanol, washed in PBS and mounted under a coverslip in a drop of 90% glycerol/PBS before inspection and counting with an epi-illuminated ultraviolet light microscope. Total lymphocyte counts were made on Giemsa-stained blood smears. T_H and T_S refer respectively to helper and suppressor T lymphocytes. NS, not significant. Paired *t* tests were used to calculate *P* values.

known to be decreased in *P. falciparum* malaria¹⁵, perhaps due to a lack of T_H help.

Our findings are supported by data from elsewhere. In Papua New Guinea, where Burkitt's lymphoma is endemic, residents in areas holoendemic for malaria were found to have impaired EBV-specific T-cell immunity compared with residents in non-malarious areas¹⁶. In Sweden, patients with primary *P. falciparum* infections had a lower proportion of T_H cells and a lower T_H/T_S ratio than normal Swedes¹⁷.

Low T_H/T_S ratios are found in a variety of infections, notably acquired immune deficiency syndrome¹⁸, where a human T-cell lymphotropic virus infects T-helper cells¹⁹, resulting in severe immunodepression and greatly increased spontaneous immunoglobulin production by B cells⁸. Could malaria activate a latent virus causing T_H-cell destruction, loss of control of B cells infected with EBV and spontaneous immunoglobulin production? The loss of T_H cells was not due to cytotoxic antibodies generated during malaria, for we (data not shown) and others²⁰ have found minimal killing of lymphocytes by acute-phase malaria serum when incubated with complement at 37 °C.

The fact that B cells transformed by EBV are capable of producing large amounts of *P. falciparum* antibody that inhibits the growth of the parasite²¹, as well as specific antibody to the virus and heterophilic antibody, is of interest. Children destined to develop Burkitt's lymphoma have abnormally high titres of antibody to viral capsid antigen²², which might be a signal that large numbers of their B cells are infected with the virus. During repeated attacks of malaria, as a result of loss of T-cell control, these cells would proliferate, increasing the number of cells in which chromosome translocation, and ultimately malignancy, may occur. Another indirect effect of the disturbances set in train by malaria may be that the latent virus, once out of control, switches on immunoglobulin genes in infected cells, thereby producing large amounts of immunoglobulin and making translocations to chromosomes on which these loci lie more likely and frequent. The end results are high serum immunoglobulin levels and an increased frequency of Burkitt's lymphoma following repeated bouts of immunosuppression by the parasite.

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Mucosal mast cells are functionally active during spontaneous expulsion of intestinal nematode infections in rat

R. G. Woodbury*, H. R. P. Miller†§, J. F. Huntley†, G. F. J. Newlands†, A. C. Palliser‡ & D. Wakelin‡

* Department of Biochemistry, University of Washington, Seattle, Washington 98122, USA

† Department of Pathology, Moredun Research Institute, 408 Gilmerton Rd, Edinburgh EH17 7JH, UK

‡ Department of Zoology, University of Nottingham, Nottingham NG7 2RD, UK

Infestation of the gastrointestinal tract by parasitic nematodes is invariably associated with mucosal mastocytosis^{1,2}, which is a thymus-dependent phenomenon in parasitized rats³, and is adoptively transferable with a T cell-enriched population of thoracic duct lymphocytes⁴. When derived by *in vitro* culture, mucosal mast cells (MMC) arise from a bone marrow precursor after stimulation by T cell-derived factors⁵. In rats infected with the nematode *Trichinella spiralis*, mucosal mastocytosis is temporally associated with the immune expulsion of the adult worms⁶ whereas in the case of *Nippostrongylus brasiliensis*, mastocytosis is frequently observed to occur after worm expulsion has been completed^{7–9}.

§ To whom correspondence should be addressed.

Consequently, there has been doubt as to whether MMC are active and serve a functional role in the expulsion of rat intestinal nematodes. MMC contain and secrete a neutral proteinase, rat mast cell protease II (RMCP II)¹⁰⁻¹³; detection and assay of secreted RMCP II therefore provides a direct measurement of MMC activity. Here we describe the release of this enzyme into the blood of rats infected with *N. brasiliensis* or *T. spiralis*. Our results show that the systemic secretion of RMCP II coincides with the immune expulsion of these nematodes, demonstrating clearly for the first time that rat MMC are functionally active during the immune elimination of primary nematode infections.

Following oral infection of PVG rats with 1,500 *T. spiralis* muscle larvae, most adult worms were expelled from the intestine between 9 and 12 days after infection (Fig. 1), elimination being complete by day 15. Mucosal mastocytosis commenced on day 6 and was maximal at day 12 (Fig. 1). No RMCP II was detected in sera either from uninfected controls or from rats infected for 3 days. By day 6, however, significant ($P < 0.05$; Mann-Whitney U test) concentrations of this enzyme were present in the serum (Fig. 1) and these reached a peak of $4.3 \pm 0.6 \mu\text{g RMCP II ml}^{-1}$ on day 12 of infection (Fig. 1). Serum levels of RMCP II had declined to $2 \mu\text{g ml}^{-1}$ by day 15 and sera were negative for the enzyme 22 days after infection (Fig. 1).

In a second experiment, adult *N. brasiliensis* worms were expelled 10-12 days after subcutaneous infection of Wistar rats with 3,000 third-stage larvae (Fig. 2). Maximal MMC hyperplasia occurred on day 12 (Fig. 2) but, in contrast with infection by *T. spiralis*, this was preceded, on day 6, by a significant ($P < 0.001$, Student's *t*-test) depletion of MMC associated with a significant ($P < 0.001$, Student's *t*-test) reduction of the concentration of RMCP II in jejunal tissues (Fig. 2). These results agree with previous studies^{12,14} and may reflect the release from adult worms of a mast cell degranulating agent¹⁵. The MMC present 9, 10 and 11 days after infection were predominantly immature^{16,17} and their numbers increased during this period, as did the concentration of RMCP II in the jejunal mucosa (for day 6 versus day 9, $P < 0.001$; Student's *t*-test) (Fig. 2). However, whereas mucosal mastocytosis was maximal on day 12, maximal accumulation of RMCP II was not observed in the jejunal mucosa until day 13 (Fig. 2). No RMCP II was detected in sera from either uninfected controls or rats infected for 6 days (Fig. 2), but between days 9 and 11 approximately $0.3 \mu\text{g RMCP II per ml}$ of serum was detected, and the concentration rose to $0.5 \mu\text{g ml}^{-1}$ on days 12 and 13 (Fig. 2).

Because, in the preceding experiment, the serum levels of RMCP II were still elevated 13 days after challenge when most worms had been expelled, a further group of rats was infected with 5,000 *N. brasiliensis* larvae, and the systemic secretion of protease was measured daily between days 6 and 16 after infection. Again, no RMCP II was detected in sera obtained before infection (day 0), but on this occasion there were trace amounts ($119 \text{ ng per ml serum}$) on day 6, and the peak of the response ($1.1 \mu\text{g per ml serum}$) occurred on day 10 (Fig. 3). Between 12 and 16 days after infection, the concentration of RMCP II in the serum fluctuated around 200 ng ml^{-1} (Fig. 3). The immune response, as judged by faecal egg counts, became effective on day 9 of infection and egg counts fell to zero on day 11, which is consistent with major worm expulsion occurring between days 10 and 12 and, compared with worm counts on day 9, there was a reduced worm burden on day 10 and parasite expulsion was complete by day 16 (Table 1). The patterns of accumulation of immature MMC and RMCP II in the jejunum (Table 1) were similar to those described in the preceding experiment (Fig. 2).

In marked contrast with the prominent mastocytosis occurring in the jejunum, the numbers of MMC in the ileum increased only gradually (Fig. 2), there being no significant increase between days 6 and 12, the period during which RMCP II was released systemically (Figs 2, 3). The slow rate of mast cell accumulation in the ileum therefore makes it unlikely that MMC in the latter are a major source of secreted enzyme. That the gut is the major source of RMCP II released into the circulation

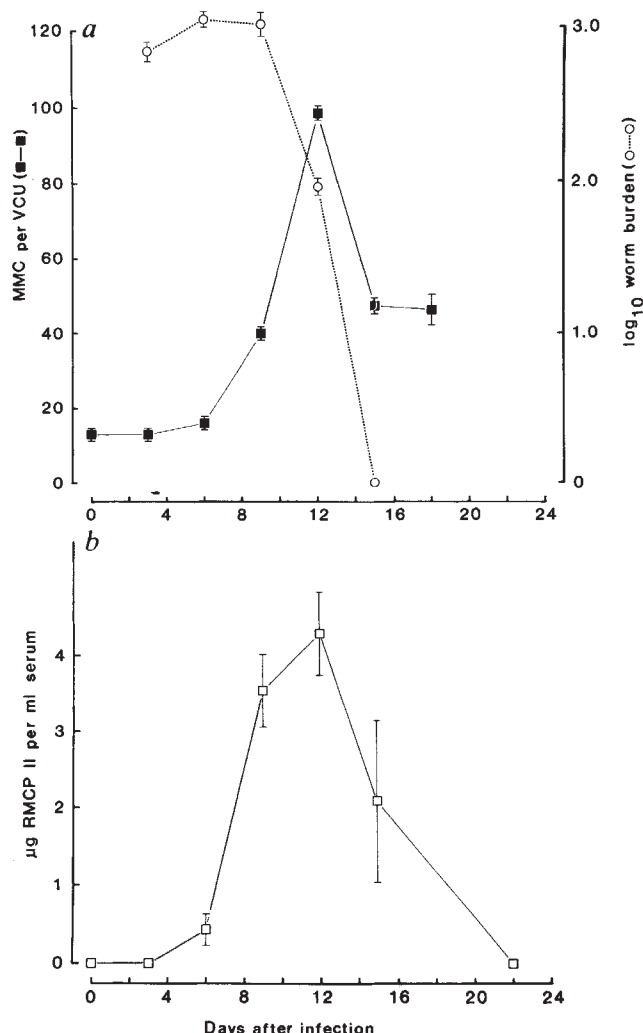


Fig. 1 *a*, Intestinal worm burden (○) and mucosal mast cell counts (■) in the jejunum of rats parasitized with *T. spiralis*. *b*, Concentration of RMCP II (□) in the sera of naive (day 0) and *T. spiralis*-infected rats (vertical bars represent $\pm$ s.e.m.). Twenty-five adult females (8-10 weeks old) were infected orally with 1,500 *T. spiralis* muscle larvae, using standard parasitological techniques³⁰. Rats (3-5 animals per group) were killed at intervals to determine worm burdens and to recover serum samples. Worms were collected by incubating intestines in saline (37 °C) using a modified Baermann apparatus³⁰. Samples of jejunum were fixed in Carnoy's fluid and processed for embedding in paraffin wax. Sections (5 µm) were stained with Alcian blue/safranin and MMC were enumerated per villus/crypt unit (VCU)¹²; at least 30 units were counted for each sample. The concentration of RMCP II in serum was assayed by enzyme-linked immunosorbent assay (ELISA)¹³. Serum and tissue samples from four uninfected control rats were also processed and assayed as described above.

Table 1 Mucosal mast cell response to infection with *N. brasiliensis*

Day of infection	Worm burden	ng RMCP II per ml serum	Jejunal RMCP II ($\mu\text{g g}^{-1}$)	MMC per villus/crypt unit
6	$4,217 \pm 389$	119 ± 55	16.5 ± 10.7	0.6 ± 0.1
9	$4,384 \pm 521$	956 ± 175	28 ± 3	1.4 ± 0.6
10	$3,109 \pm 443^*$	$1,135 \pm 207$	30 ± 4	$4.6 \pm 1^\dagger$
16	0	166 ± 21	$1,755 \pm 256$	65.5 ± 6.0

Details of the experiment are given in Fig. 3 legend. The techniques for quantifying worm burdens, assaying RMCP II in jejunum and serum, and for counting MMC were those described in legends to Figs 1-3. Values are mean $\pm$ s.e.m. * $P < 0.05$; $^\dagger P < 0.001$ on day 10 versus day 9 (Student's one-tailed *t*-test).

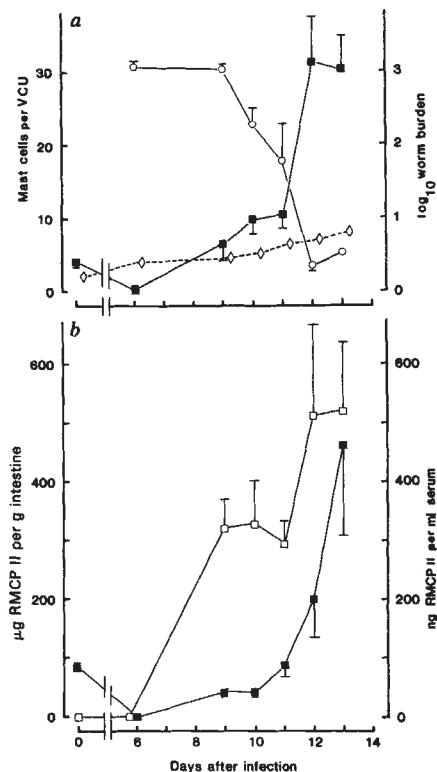


Fig. 2 *a*, Intestinal worm burden (○) and mucosal mast cell counts in the jejunum (■) and ileum (◇) of rats parasitized with *N. brasiliensis*. *b*, Concentration of RMCP II in the serum (□) and jejunum (■) of naive (day 0) and *N. brasiliensis*-infected rats (vertical bars represent $\pm$ s.e.m.). Fifty-seven 6-week-old female Wistar rats were infected subcutaneously with 3,000 *N. brasiliensis* third-stage larvae³¹. Groups comprising 5–6 rats each (four rats only on day 13) were killed at intervals and adult parasites were recovered by allowing the worms to migrate from the intestinal mucosa into saline at 37 °C (ref. 31). Segments of jejunum and terminal ileum were placed in Carnoy's fixative and processed for MMC counts as described in Fig. 1 legend. Lengths (3 cm) of jejunum were examined for the presence of parasites and were washed exhaustively, blotted dry, and weighed before they were homogenized in 3 vol. of 0.15 M KCl. Homogenates were centrifuged (10,000g) and the supernatants assayed for RMCP II by radial immunodiffusion¹². The levels of enzyme in serum were determined by ELISA as described in Fig. 1 legend. Samples from five uninfected control rats (day 0) were processed and assayed in exactly the same way.

was suggested by a recent study which showed that this enzyme is secreted in large amounts into the intestinal lumen during systemic anaphylaxis¹⁸; there was a simultaneous release of RMCP II into the circulation, together with depletion of both MMC and RMCP II from the jejunal mucosa^{13,18}.

The rate of accumulation of RMCP II in the mucosa of *N. brasiliensis*-infected rats lagged behind the development of mastocytosis, in agreement with previous reports which suggested that MMC might secrete rather than store enzyme¹² and monoamines¹⁹. The fact that MMC were immature at the time of maximal enzyme release and that, ultrastructurally, immature MMC have apparent protein synthesizing capacity²⁰, again suggests a secretory function for these cells. Further support for this role is provided by the *in vitro* behaviour of cultured MMC which, during the earliest stages of differentiation, release RMCP II into the culture medium²¹. Immature rat peritoneal mast cells actively secrete histamine²², and it is now clear that *N. brasiliensis* infection in mice generates histamine-producing cell-stimulating factor (HCSF)²³, a lymphokine probably identical to interleukin-3, which recruits putative mast cell precursors to sites of immuno-inflammatory reactions²⁴. Taken together, these studies indicate that immature mast cells and their precursors may be as functionally active as the fully granulated mature

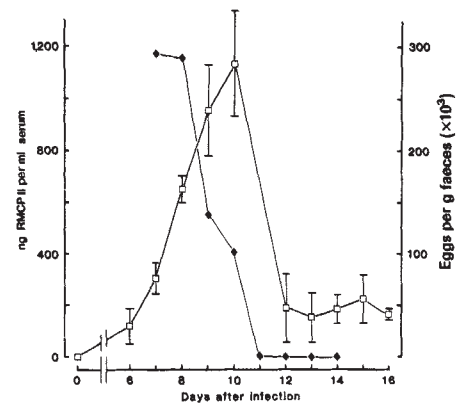


Fig. 3 Faecal egg counts (♦) and concentration of RMCP II in the sera (□) of rats infected with 5,000 *N. brasiliensis* third-stage larvae. The techniques used were similar to those described for Fig. 2 although, in this instance, 22 adult male Wistar rats were infected. A group of five rats was bled via the tail vein on the day of infection (day 0). Groups of 6–8 rats were bled on day 7 and daily thereafter (except day 11), again via the tail vein except those rats exsanguinated on days 6, 9, 10 and 16 after infection. The immune response to the parasite was monitored by faecal egg counts (bulk samples) using standard methods³¹. Concentrations of RMCP II in the sera of control (day 0) and infected rats were assayed by ELISA as described for Fig. 1.

cell, and the present results are compatible with such a possibility.

Our findings indicate for the first time a functional role for MMC during the immune expulsion of intestinal nematodes. Release of MMC mediators may allow translocation, into the gut lumen, of serum antibodies and complement by altering mucosal permeability¹. In addition, these mediators may directly affect the parasites themselves²⁵, may alter epithelial integrity¹⁸ and secretory activity²⁶, and may act on intestinal smooth muscle²⁷. The mechanisms by which MMC are activated, in the absence of detectable parasite-specific IgE^{28,29}, remain to be elucidated.

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Capacitance measurements reveal stepwise fusion events in degranulating mast cells

J. M. Fernandez, E. Neher & B. D. Gomperts*

Department of Membrane Biophysics, Max-Planck-Institut für Biophysikalische Chemie, D-3400, Göttingen, FRG

* Department of Experimental Pathology, University College London, University Street, London WC1E 6JJ, UK

Mast cells undergo an extensive and violent morphological transformation on stimulation¹. Here we describe the dynamics of fusion of the secretory granules in individual mast cells during exocytosis. The cell membrane capacitance (proportional to the cell surface area) was measured using the whole-cell patch-pipette technique^{2,3}, in which the intracellular space is dialysed with the solutions used to fill the patch pipette. Our results show that degranulation occurs spontaneously and reproducibly if the GTP analogue, GTP- γ -S, and Mg-ATP are present in the pipette filling solution. Contrary to previous reports⁴⁻⁸, in these conditions Ca^{2+} (and/or Ca^{2+} buffers) is not required for degranulation. Although electrogenic Ca^{2+} entry was not detected before or during degranulation and membrane conductance remained low, the capacitance, and by implication the area of the membrane of degranulating cells, increased sigmoidally and stepwise. We conclude that stepwise increases of capacitance are due to the fusion of individual secretory granules with the plasma membrane, and that guanine nucleotide regulatory proteins are involved in the control of this process.

To our surprise, especially in view of an earlier report⁹ indicating stimulation of exocytosis following microinjection of Ca^{2+} , perfusion with various combinations of Ca^{2+} and EGTA (to maintain cytosolic free Ca^{2+} in the range 0.2–10 μM) generally failed to induce degranulation in rat mast cells that were otherwise normal. Degranulation of neighbouring cells could be easily induced by adding compound 48/80 in their vicinity, while the dialysed cell remained unaffected.

It has recently been demonstrated that guanine nucleotide regulatory proteins are essential for the transmission of signals controlling many cellular processes¹⁰ and that a GTP-binding protein is involved in the activation of rat mast cells¹¹. When rat mast cells were dialysed through the patch pipette with solutions containing GTP- γ -S at concentrations down to 5 μM , they rapidly degranulated (Fig. 1), even in nominally Ca^{2+} -free solutions. Figure 1a illustrates the appearance of a normal mast cell viewed with Nomarski optics. A glass pipette was pressed and sealed against the cell surface using standard patch-clamp procedures¹². Following a strong suction pulse, the membrane patch enclosed by the pipette ruptured without altering the seal between the glass pipette and the rest of the cell membrane. From this point, the cell was rapidly dialysed—most ions and low molecular weight solutes will equilibrate between the cytoplasm and the effective infinite volume of the pipette within seconds¹³. After a variable lag time, degranulation occurred (Fig. 1b).

The rapid fusion of a large number of secretory granules with the plasma membrane of mast cells produced an increase in the area of the plasma membrane through the formation of large irregular cavities which transformed the morphology of the cell surface (see Fig. 1b). The changes in membrane area were monitored by measuring the cell membrane capacitance (see Fig. 1 legend); a typical protocol for these experiments is illustrated in Fig. 1c. At the start of whole-cell recording (arrow), the capacitance increased markedly (6 pF); this level corresponds to the capacitance of the membrane of the resting cell. After a variable lag time, the cells degranulated, generating a large increase in total membrane capacitance (average 3.9 ± 0.9 -fold, $n = 12$), and reached a new steady state ~3–5 min after the onset of degranulation.

Similar results were obtained when experiments of the type

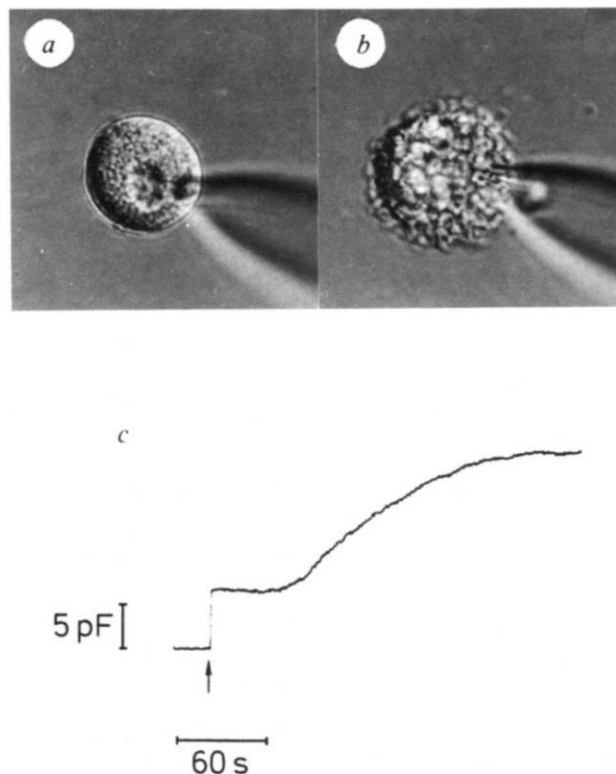


Fig. 1 Effect of dialysis of a single rat peritoneal mast cell with pipette solutions containing GTP- γ -S. **a**, A typical mast cell observed with Nomarski optics on a Zeiss inverted microscope (IM35). The patch pipette can be seen slightly out of focus, coming in at an angle at the lower right-hand corner. This picture was taken after formation of a giga-seal in the cell-attached mode. Typical features of mast cell morphology can be observed at this time, in particular a prominent nucleus and a cytoplasm densely packed with secretory granules. **b**, The same cell photographed ~5 min after the start of dialysis and whole-cell recording. Extensive changes in appearance, corresponding to exocytotic degranulation, can be seen: residues of intragranular matrix material are floating near the cell. The composition of the pipette-filling solution was: 10 μM GTP- γ -S (Boehringer-Mannheim), 100 μM Na-EGTA, 1 mM ATP, 150 mM K-glutamate, 7 mM MgCl_2 , 10 mM HEPES. The external medium comprised (mM): 150 NaCl, 2.8 KCl, 1 MgCl_2 , 6 CaCl_2 , 10 HEPES. All solutions were adjusted to pH 7.2 with NaOH and the osmolality was maintained at 300–320 mosmol kg^{-1} . **c**, A large increase in the membrane area of degranulating mast cells can be measured at the same time as the morphological changes are being observed under the microscope. Changes of area were monitored by measuring the cell membrane capacitance at low frequencies (414 Hz) using the synthetic Pseudo Random Binary Sequence (PRBS) method described by Fernandez *et al.*³. An 18-mV PRBS (128 bits long, 6,621 Hz total bandwidth) was continuously applied to the command input of an EPC-7 patch-clamp (List Electronics). In voltage-clamp conditions (typically 0 mV), the whole-cell membrane current in response to the PRBS stimulus was sampled and stored by a PDP-11/23 minicomputer (Digital Equipment Corporation) operating on-line. The membrane capacitance was then evaluated from the imaginary part of the cell membrane admittance. This method allows accurate determination of the linear parameters of the cell—d.c. capacitance, d.c. conductance and series resistance—over wide ranges as the cell degranulates. The figure shows the time course, before and during degranulation, of the cell membrane capacitance. This was measured in the whole-cell recording mode following identical procedures to those used for **a** and **b** (internal solution contained GTP- γ -S at 50 μM in this case). The arrow indicates the time of transition from cell-attached to whole-cell recording, at which point the contribution of the cell membrane capacitance becomes apparent (typically 5–6 pF). After an initial delay, the cell degranulated and the capacitance increased 3–6-fold. Membrane resistance (not shown) did not change significantly during degranulation. All experiments were carried out at room temperature.

shown in Fig. 1 were repeated using external media in which Ca^{2+} was maintained at submicromolar concentrations (50 μM EGTA and no added Ca). This suggests that extracellular Ca^{2+} is not a requirement for degranulation in the conditions we used. Indeed, the cell membrane conductance remained at a very low level during degranulation and any increases observed (measured in four cases, 180 ± 150 pS) were always very small. This contrasts with the situation pertaining in adrenal chromaffin cells in which exocytosis can be induced by inward Ca^{2+} currents of only ~ 100 pA (in the range 1,000–2,000 pS (ref. 2)).

An estimate for the maximal increment in membrane capacitance can be obtained from stereological electron microscopic observations¹⁴ of the diameter distribution of the secretory granules of rat peritoneal mast cells. According to these data, cells, typically 10.9 μm in diameter, will contain 1,020 secretory granules of ~ 0.77 μm diameter, thus predicting an increase in capacitance from 3.73 pF for the resting cell to 18.96 pF for a cell in which all granules have fused with the plasma membrane. Deviations from this estimate are to be expected because of variation in granule size, and these should be taken into account. Also, in our experiments we have used larger cells (average diameter measured in 15 cells was 12.8 ± 1.4 μm , giving a capacitance of 5.2 pF in the resting state), containing a larger number of secretory granules. Even taking this into consideration, our estimate shows that the largest capacitative changes we have observed can only be explained by postulating that most of the secretory granules fuse with the plasma membrane.

The presence of large secretory granules in rat peritoneal mast cells, and their fusion with the plasma membrane on stimulation, predicts that the increases of capacitance observed in Fig. 1c should occur in a stepwise manner as single granules fuse with the plasma membrane. This is illustrated in Fig. 2 and is consistent with the concept of granule fusion and retrieval as being the mechanism of exo- and endocytosis. Cells degranulate rather slowly at first, after an initial delay (Fig. 1c). When this is observed with high gain, the capacitance is seen to increase in a stepwise manner ('on' steps, Fig. 2a).

We have observed two other types of discrete capacitance changes. Occasionally, particularly when we used filling solutions lacking the GTP analogue and containing high concentrations of EGTA (0.5 mM), the capacitance declined in steps over a period of 5–10 min (Fig. 2b). It is likely that in such situations some degree of degranulation had occurred during the establishment of the whole-cell recording configuration, so that the step decreases of capacitance ('off' steps) represent retrieval of excess membrane. Episodes were also occasionally observed during which the capacitance fluctuated transiently between two levels ('flicker'; Fig. 2c). Although we did not study these two phenomena extensively, we consider that they are probably related to granule fusion and retrieval, as they are not associated with conductance changes of the kind produced by the switching of ionic channels.

Further evidence that the data of Fig. 2 are indeed related to granule fusion and retrieval (exo- and endocytosis) was obtained by comparing the amplitude distribution of these processes. Figure 3a, b shows the amplitude distribution for the on and off steps, respectively. The expanded-scale distributions (Fig. 3a, b, lower panels) show good agreement between the magnitude of most of the capacitance steps and the distributions predicted on the basis of the dimensions of the secretory granules. Note, however, the occurrence of a population of capacitance steps (> 60 fF) which are too large to be accommodated by the fusion or retrieval of individual granules. These larger events are likely to correspond to the fusion or retrieval of multiple fused vesicles, corresponding to what has been described as compound exocytosis¹⁵.

Figure 3c shows the size distribution of capacitance flicker. The large steps are absent from this distribution, and all events fall within the range expected for single vesicle fusion events. Models proposed for exocytosis typically consider vesicle fusion with the plasma membrane to be an irreversible process. In contrast, an attractive way to explain the flicker we have observed

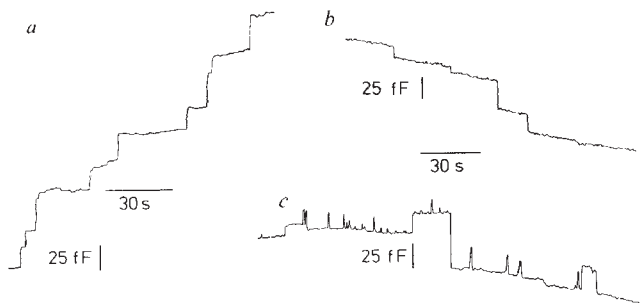


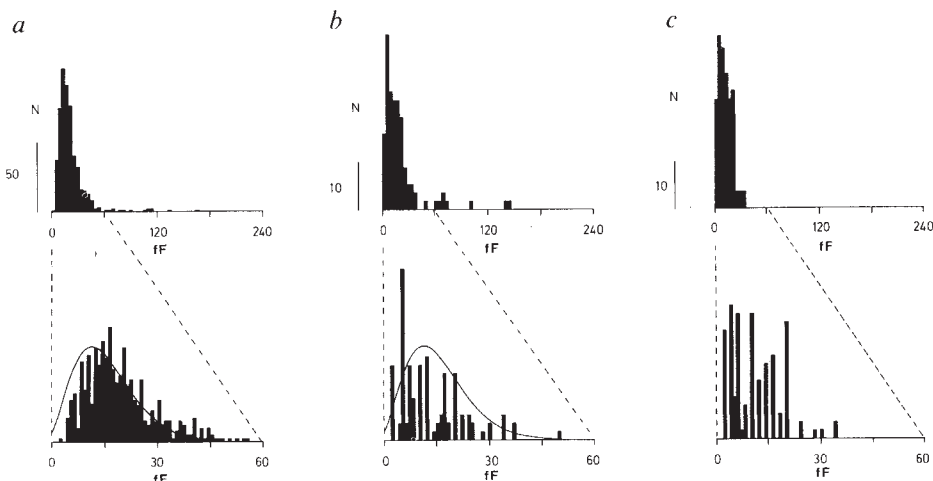
Fig. 2 Stepwise changes in the capacitance of mast cells undergoing exocytotic degranulation. **a**, Typical time course of capacitance changes monitored at the start of degranulation in a rat mast cell dialysed internally with GTP- γ -S. Capacitance increments associated with degranulation occur in a stepwise fashion, in agreement with the concept of vesicle fusion. The step increases ('on' steps) shown are not associated with measurable changes in membrane conductance and occur independently of the membrane potential. Electrode filling solution comprised: 140 mM KCl, 7 mM MgCl_2 , 5 μM GTP- γ -S, 200 μM ATP, 375 μM EGTA, 320 μM CaCl_2 . This particular internal filling solution caused a slow, partial degranulation. (ATP concentrations were kept as low as possible so as to reduce the possibility of ATP leakage from the patch pipettes while they were being brought into contact with mast cells. This would have caused solute permeability lesions to form in the plasma membrane with consequent unwanted effects¹⁷.) **b**, Stepwise decreases of capacitance ('off' steps) measured on cells dialysed with solutions containing no GTP- γ -S and a relatively high Ca^{2+} buffering capacity. In these conditions, the cell capacitance typically falls and this also occurs in a stepwise fashion. Electrode filling solution comprised: 130 mM K-glutamate, 20 mM Na-glutamate, 7 mM MgCl_2 , 10 mM HEPES, 5 mM ATP, 100 μM EGTA, pH 7.2. **c**, Capacitance 'flicker'. Transient increases of capacitance of relatively short duration, usually absent from downward trends like those of **b**. The capacitance flicker can be observed in both the presence and absence of GTP- γ -S, and/or various combinations of Ca^{2+} /EGTA. In the experiment illustrated, EGTA was omitted from the electrode filling solution which was otherwise the same as for **b**. In these experiments, the external buffer solution was identical to that used for the experiments of Fig. 1c except that the concentration of Ca^{2+} was 2 mM.

Methods: Capacitance steps of the kind expected for the fusion of a single secretory vesicle are best measured by the phase-sensitive detection method of Neher and Marty², which is less noisy than the PRBS method used in the Fig. 1c experiment. It cannot, however, track large capacitance changes of the type shown in Fig. 1c, as the method works well only for small deviations from the compensated cell capacitance. In this figure, capacitance was tracked with an 11-mV sinusoidal signal of 800 Hz. Simultaneously, the quadrature component (membrane conductance) was monitored with a second phase-sensitive detector tracking the sinusoidal signal at 90° with respect to the capacitance detector. The conductance as monitored in this way did not seem to be correlated with the capacitance events illustrated, showing only the occasional opening of a single ion channel or nonspecific leakage (see text).

is to assume the formation of a reversible aqueous pore between the secretory vesicle and the plasma membrane, before formation of the (irreversible) fused state. Alternatively, newly fused vesicles might be pinched off by the strong mechanical disturbances that must occur during degranulation, as vesicles fusing with each other and with the plasma membrane are known to cause extensive reorganization of the cell surface¹⁶.

Our results provide a way of monitoring quantitatively the kinetics of granule fusion (and release) in a single cell. An advantage of our approach is the simultaneous dialysis of the cytoplasm with the solutions used to fill the pipette, which enables pipette biochemistry to be carried out concomitantly with the electrical measurements. Our results are surprising in that they show that the introduction of the GTP analogue by dialysis through the patch pipette is a sufficient stimulus to secretion even in conditions where Ca^{2+} is absent both from the

Fig. 3 Size distribution (N) of 'on' (a) and 'off' (b) steps, and capacitance 'flicker' (c). Measurements were collected from 25 different cells in experiments similar to those of Fig. 2a (see Fig. 2 legend for details of conditions). **a**, On step distribution obtained mainly during the earliest degranulation events at the start of secretion and during subsequent phases of slow degranulation in the conditions of Fig. 2a. The expanded scale (lower panel) shows the agreement between the observed step size and that predicted by the vesicle diameter frequency distribution (continuous line) obtained with electron microscopic methods¹⁴. The data for the vesicle diameters were fitted by a normal gaussian distribution having a mean of $0.68 \pm 0.21 \mu\text{m}$ ($\pm$ s.d.). Using these parameters, the capacitance step distribution for fusion of individual granules was predicted as shown and found to agree well with our data. This distribution also predicts that no steps larger than 60 fF should be observed. The large on steps probably correspond to the fusion with the plasma membrane of clusters of previously fused granules (compound exocytosis). **b**, Off step size distribution obtained from non-degranulating cells. The expanded scale (lower panel) once again shows good agreement between the data and the predicted size distribution (same parameters as in a). At small sizes the data deviate significantly from the predicted distribution and this may reflect two types of endocytotic processes occurring simultaneously. **c**, Flicker size distribution. Note the absence of very large sizes as opposed to the data shown in a and b. The expanded scale (lower panel) shows that the flicker size distribution is confined to the limits predicted by single vesicle sizes. We have not attempted to fit these distributions.



pipette and the external solutions. This contrasts with the earlier finding that GTP analogues introduced into mast cells that have been reversibly permeabilized with low concentrations of ATP⁴⁻ causes them to undergo exocytotic secretion of histamine only on subsequent provision of extracellular Ca²⁺ (ref. 11).

The differing requirements for Ca²⁺ in the various experimental systems probably depend on the degree of dialysis achieved. For instance, the conflict can be adequately resolved by postulating that a Ca²⁺-dependent inhibitory protein present in the cytosol of intact cells prevents the direct activation of exocytosis by the GTP analogue. After the loss of this protein through the very efficient dialysis performed with the patch pipette, the analogue might act in a Ca²⁺-independent manner. In intact cells and in cells loaded with GTP analogues following permeabilization with ATP⁴⁻, in which the full complement of soluble proteins is retained, direct action by the nucleotide is prevented by this inhibitory protein. Elevation of intracellular Ca²⁺ would act to remove this inhibition.

Although the site and mode of action of the GTP analogue remains unknown, it seems logical to assume that it binds to a guanine nucleotide regulatory protein, thus activating an as yet unknown chain of events leading to the massive release of histamine and other secreted products. Possible sites for such regulatory proteins are the plasma membrane¹¹, the endoplasmic reticulum and the secretory granule membrane.

The δ - and ϵ -chains of the human T3/T-cell receptor complex are distinct polypeptides

Jannie Borst, John E. Coligan*, Hans Oettgen, Silvana Pessano, Robert Malin & Cox Terhorst

Laboratory of Molecular Immunology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts 02115, USA

* Laboratory of Immunogenetics, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20205, USA

The T3/T-cell receptor complex on the surface of human thymus-derived lymphocytes consists of four glycoproteins: the α -chain of relative molecular mass (M_r) 40,000–50,000 (40–50K), the β -chain (37–45K); the γ -chain (25K) and the δ -chain (20K)^{1–3}. The T3 α - and β -chains have been identified as clonotypic T-cell receptors^{3–7}, but functionally the T3/T-cell receptor chains seem to form a single complex: monoclonal antibodies directed at the 20K T3 components are mitogenic for normal human T lymphocytes^{8,9} and, at higher concentrations, anti-clonotypic and anti-20K reagents block T-cell function^{4,10}. Recently, Zanders *et al.*¹¹ showed that incubation of human T-helper clones with high concentrations of antigen abolishes antigen-specific proliferation and induces disappearance of T3 from the cell surface. Thus, the T3/T-cell receptor complex consists of two variable subunits, the T3 α - and β -chains, which interact with antigen and the monomorphic 20K/25K T3 chains. Recently, the existence of a fifth polypeptide chain, the unglycosylated T3 ϵ -chain, has been postulated^{12,13}. Here we confirm that a 20K ϵ -chain does exist. The T3 ϵ -chain differs from the T3 δ -chain in primary structure as judged by N-terminal amino acid sequencing, peptide mapping and immunoblotting with anti-T3- δ and anti-T3- ϵ antibodies. Treatment with endoglycosidase F revealed two nonglycosylated T3 δ polypeptide backbone chains (16K and 14K) with identical amino termini. Together with previous pulse-chase experiments² this observation suggests that the 14K T3 polypeptide is derived from the 16K T3 precursor by proteolytic processing near the C-terminus of the molecule.

Using differential labelling studies and pulse-chase experiments, we suggested previously that two 20K T3 components might exist¹². We had found that one 20K polypeptide chain

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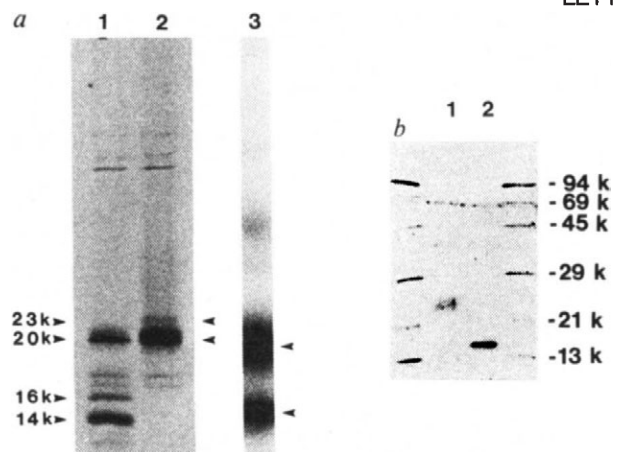


Fig. 1 Purification of the T3 δ - and ϵ -chains for amino acid sequencing. *a*, Radiolabelled 20K T3 proteins: lane 1, SDS-PAGE analysis of radiolabelled T3 proteins after Endo-F treatment; lane 2, SDS-PAGE analysis of radiolabelled T3 proteins before Endo-F treatment; lane 3, ^{125}I -labelled, Endo-F-treated 20K T3 material. *b*, Large-scale purification: lane 1, purified 20K T3 polypeptide chain; lane 2, purified 14K T3 polypeptide chain. Numbers on the right-hand side are *M_r* markers.

Methods: *a*, HPB-ALL cells were labelled with ^3H -amino acids and ^{35}S -L-methionine (or ^{35}S -L-cysteine) as described elsewhere²⁰. Cells were washed four times in RPMI medium without amino acids and resuspended at a concentration of 5×10^6 cells per ml in the same medium containing 5% fetal calf serum and (aminoxy)acetic acid. Lyophilized amino acids redissolved in RPMI without amino acids were then added to the cells and incubation proceeded for 10 h at 37 °C in an atmosphere of 5% CO_2 . The cells were collected by centrifugation at 300g for 10 min and prepared for immunoprecipitation^{2,23}. Immunoprecipitates were treated with Endo-F² and the samples were run on SDS-PAGE on gradient gels (10–15% in acrylamide). The gels were dried, autoradiography performed and slices were then excised and eluted by the addition of 1 ml 10 mM NH_4HCO_3 and 0.01% SDS in the presence of 200 μg sperm whale myoglobin (Beckman). After 12 h incubation at 37 °C in the presence of protease inhibitors, the eluate was passed through nylon wool and dialysed against two changes of 500 ml of 10 mM NH_4HCO_3 at 4 °C, followed by lyophilization. *b*, HPB-ALL cells were cultured at the MIT Cell Culture Facility in RPMI 1640 medium, supplemented with 5% fetal calf serum. For purification of T3, $\sim 50 \times 10^9$ cells were used. Cells were disrupted by nitrogen cavitation and a membrane fraction was prepared²⁴. Membranes were solubilized in 0.01 M triethanolamine pH 7.8, 0.15 M NaCl, 2% Nonidet P40, 1 mM EDTA, 1 mM phenylmethylsulphonyl fluoride, 25 mM iodoacetamide; the extract was prepurified by passage through two columns consisting of murine IgG-coupled protein-A-Sepharose (Pharmacia) and through a wheat germ agglutinin-Sepharose column (Vector T3). T3 was isolated on an immunoadsorbent column, made with the T3 monoclonal antibody anti-Leu-4 (Becton-Dickinson nomenclature), coupled to protein-A-Sepharose²⁵. This column was subjected to several washes²³ before elution with 3.5 M MgCl_2 in the above buffer. Protein was recovered from the eluate by precipitation with trichloroacetic acid. Further purification was achieved by SDS-PAGE: the 20K T3 material was eluted from the gel as described previously²⁶ and subjected to digestion by Endo-F. A radiolabelled sample was analysed on a 10–15% SDS-PAGE gel (*a*, lane 3). The 14K and 20K T3 components were isolated by SDS-PAGE and electroelution²⁶. Purified 20K T3 (lane 1) and 14K T3 (lane 2) were analysed on a 'mini gel'²⁷ and stained with Coomassie brilliant blue.

(T3 δ -chain) is susceptible to treatment with endo- β -N-acetylglucosaminidase F (Endo-F) whereas the other is not (T3 ϵ -chain)¹². Structural differences between the T3 δ - and T3 ϵ -chains were assessed by determining their N-terminal amino acid sequences. For this, cells from two unrelated T-cell lines, HPB-ALL and JM, were labelled for 8 h with ^{35}S -L-methionine or ^{35}S -L-cysteine and one other amino acid labelled with tritium. In each experiment, an immunoprecipitate was made with a monoclonal antibody to T3 and the various T3 chains were isolated by preparative SDS-polyacrylamide gel electrophoresis

	1	5	10	15	20	25	30
T3- ϵ 20K	-	L	-	K	M	-	V
T3- δ 14K	F	K	I	P	E	E	L
T3- δ 16K	F	K	I	I	V	F	V
T3- δ 23K	I	I	L	V	V	C	V

Fig. 2 N-terminal amino acid sequences of the T3 δ - and T3 ϵ -chains. All sequences were obtained from protein isolated from HPB-ALL, except the 14K sequence, which was also derived from the cell line JM. — Indicates that no residue could be assigned. The 14K sequence was developed from both unlabelled and radiolabelled protein preparations. For the 16K and 23K polypeptide chains, only the amino acids shown in the figure were used in the sequencing experiments.

Methods: Radiosequencing was performed on a Beckman 890C protein sequencer with a cold trap modification using the Beckman 0.1 M Quadrol program 121078. Identification of the phenylthiohydantoin (PTH)-amino acids was as described by Coligan *et al.*²⁸. Protein sequencing on purified T3 proteins from large-scale preparations was performed on an Applied Biosystems protein sequencer, Model 470A, using the MHTFA program. PTH-amino acids were identified on reverse-phase HPLC with a Beckman 346 HPLC and a Beckman 165 detector, using an Ultrasphere ODS column (3 μm , 0.46×7.5 cm). Solvent A was 93% H_2O /7% solvent B containing 1.4 ml l^{-1} triethylamine, adjusted to pH 4.25 with acetic acid. Solvent B was isopropanol/ CH_3CN (2.5:1). The PTH-amino acids were analysed at 40 °C using the following program: 0–3.5 min, 1.5% B; 3.5–15 min, 21% B; 15 min, set for 100% B in 12 s.

(PAGE). A small amount of a 23K T3 species was detected in addition to 20K T3 (Fig. 1*b*, lane 2). Although Endo-F treatment resulted in a 14K protein, together with the remaining 20K material, a 16K digestion product was also present (Fig. 1*a*, lane 1). Experiments with the enzyme endoglycosidase H (Endo-H) and the drug tunicamycin demonstrated that this 16K species is derived from a 23K glycosylated form of T3.⁷ Pulse-chase experiments showed that this 23K species is converted to a glycosylated 20K T3 chain (ref. 2 and unpublished data).

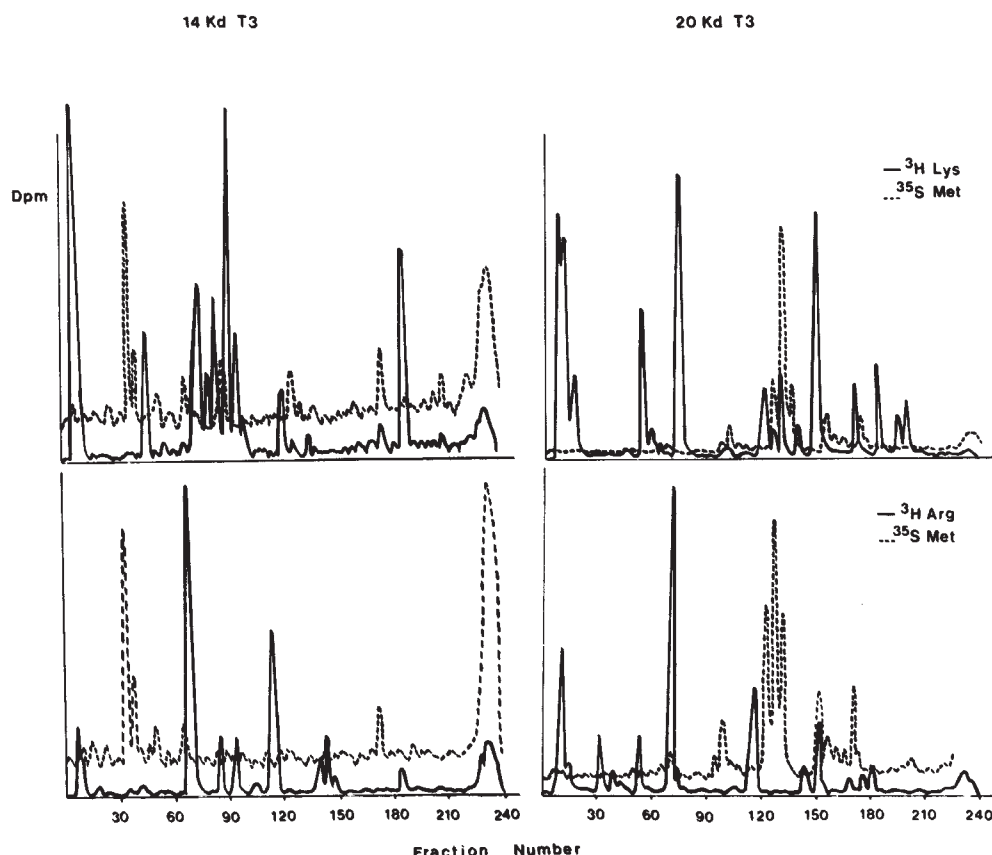
When N-terminal amino acid sequences were obtained from the 20K, 16K and 14K T3 bands after Endo-F treatment and from the 23K and 20K T3 bands before Endo-F digestion, the 23K T3 polypeptide chain and the Endo-F-treated 16K T3 polypeptide chain were found to have the same amino-terminal sequence as the deglycosylated 14K T3 δ -chain (Fig. 2). The remaining 20K T3 material which was not susceptible to Endo-F treatment (T3 ϵ -chain) had a different N-terminal amino acid sequence from the 14K T3 product (Fig. 2), whereas the 20K T3 material that had not been treated with Endo-F contained both sequences. Sequences obtained for the T3 δ -chain isolated from HPB-ALL and JM were identical.

We conclude from these experiments that: (1) the amino terminus of the 20K nonglycosylated T3 product (T3 ϵ -chain) is different from that of the glycosylated 20K T3 product (T3 δ -chain); (2) the 23K and 16K T3 components have the same N-terminus as the 14K T3 component; (3) the T3 δ -chain isolated from HPB-ALL cells has the same N-terminus as the T3 δ -chain from JM cells.

These sequencing data, together with the pulse-chase, Endo-H and tunicamycin experiments^{2,13}, provide evidence that the 23K T3 polypeptide chain (16K without oligosaccharides) may have been converted to the 20K T3 polypeptide chain (14K without oligosaccharides) by proteolysis. This proteolytic cleavage must have taken place near the C-terminus of the protein because all intermediates have an identical N-terminal sequence. The conversion from 23K to 20K during the pulse-chase experiment may therefore represent an *in vivo* proteolytic processing step, although protease contamination during the Endo-H and Endo-F experiments cannot be excluded. The notion that the 16K T3 δ -chain is the primary protein product is supported by hybrid selection and *in vitro* translation experiments with a T3 δ -chain cDNA clone (see accompanying article¹⁴).

To determine the N-terminal amino acid sequences of unlabelled T3 δ - and T3 ϵ -chains, the combined 20K T3 com-

Fig. 3 Tryptic peptide analysis of the T3 δ - and T3 ϵ -chains. The T3 δ - and T3 ϵ -chains were isolated from an anti-Leu-4 immunoprecipitate which had been treated as described for Fig. 1a (lane 1). The 14K and 20K material was either metabolically labelled with ^3H -L-lysine and ^{35}S -L-methionine or with ^3H -L-arginine and ^{35}S -L-methionine (see Fig. 1). Reduced and alkylated proteins were eluted from a preparative SDS-PAGE, dialyzed and precipitated with ice-cold acetone in the presence of 100 μg bovine γ -globulin as a carrier. The lyophilized protein was dissolved in H_2O , precipitated with acetone, resuspended in 0.05 M NH_4HCO_3 , pH 7.4, TPCK-trypsin (Millipore) in 0.001 M HCl added at a ratio of 10:1 (carrier protein:enzyme), and incubated at 37 $^\circ\text{C}$ for 4 h. The digest was lyophilized and the tryptic peptides were resolved by reverse-phase HPLC on a Supelcosil C_{18} column (150×4.6 mm i.d., Supelco) at a flow rate of 1 ml min^{-1} with a Spectra-Physics SP8000B liquid chromatograph. After initial isocratic elution for 5 min in 0.1% CF_3COOH , linear gradient elution to 0.1% $\text{CF}_3\text{COOH}/30\% \text{CH}_3\text{CH}$ was run for 95 min followed by gradient elution to 0.1% $\text{CF}_3\text{COOH}/100\% \text{CH}_3\text{CN}$ in 10 min. Fractions were collected at 0.5-min intervals and analysed by liquid scintillation counting.



ponents were isolated from large amounts of HPB-ALL plasma membrane fractions (see Fig. 1) by elution from immunoabsorbent columns and preparative SDS-PAGE; the purified protein was then radiolabelled with Na^{125}I and treated with Endo-F. Separation of the Endo-F digestion products of the 20K T3 material by SDS-PAGE revealed that they contained only the 14K form and undigested 20K protein (Fig. 1a, lane 3). This supports the idea that the 23K/16K T3 species can be found only as an intermediate in the pulse-chase metabolic labelling experiments. The purified 14K and 20K proteins (Fig. 1b) were used for amino acid sequencing in the gas-phase sequencer. An unambiguous sequence was obtained only from the 14K material and was identical to the first 15 residues found by radiolabelled sequencing (Fig. 2). This is an important confirmation of the 14K T3 sequence determined with radiolabelled amino acids because it gives a unique contiguous sequence. Inspection of the limited N-terminal sequences of the 14K T3 polypeptide chain (δ -chain) did not reveal any homology with immunoglobulins¹⁵, T3/T-cell receptor α - or β -chains¹⁶⁻¹⁹, the T-cell differentiation antigen T8 (ref. 20) or rodent Thy-1 (ref. 21).

Structural differences between the T3 δ - and ϵ -chain were investigated further by HPLC of tryptic peptides. Radiolabelled 14K and 20K T3 polypeptide chains were prepared from HPB-ALL cells that had been cultured in the presence of ^3H -lysine or ^3H -arginine. For easy detection of the T3 proteins during the purification, ^{35}S -methionine was always added to the cultures. The tryptic peptide maps obtained from the T3 δ - and ϵ -chains were clearly different (Fig. 3). This finding, together with the N-terminal sequence data, confirms the existence of two distinct 20K T3 proteins.

The T3 ϵ -chain had previously been identified by its capacity to be labelled by the hydrophobic reagents ^{125}I -5-iodonaphthyl-1-azide (^{125}I INA) (ref. 12) and ^{125}I -3-(trifluoromethyl)-3-(m-iodophenyl) deazirine (^{125}I -TID) (data not shown) and had been isolated based on its resistance to Endo-H and Endo-F. To obtain a means of positive identification of the T3 ϵ -chain, monoclonal antibodies were prepared by immunizing mice with

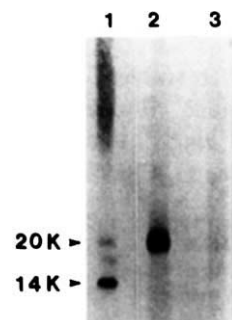


Fig. 4 Immunoblot analysis of T3 δ - and T3 ϵ -chains. Lane 1, rabbit anti-T3 δ -chain serum (6081); lane 2, monoclonal reagent SP6; lane 3, normal mouse serum.

Methods: Plasma membranes were isolated from HPB-ALL cells as described in Fig. 1 legend. Membrane proteins were treated with Endo-F and separated on a SDS-PAGE mini-gel²⁷. The proteins were transferred to nitrocellulose²⁹ and 4-mm wide strips were cut out and developed with antiserum 6081 or monoclonal antibody SP6 and ^{125}I -labelled protein A. Rabbit serum 6081 had been prepared with a synthetic peptide containing amino acids 1-14 of the T3 δ -chain (see Fig. 2 legend)³⁰. Monoclonal antibody SP6 was obtained by immunizing a BALB/c mouse with denatured 20K T3 material and selecting with purified δ - and ϵ -chains using a dot-blotting technique (S.P. *et al.*, manuscript in preparation).

purified denatured 20K T3 polypeptide chains. One of these antibodies (SP6) detected a 20K T3 polypeptide chains in an immunoblotting experiment after treatment with Endo-F (Fig. 4, lane 2). To establish the extent of hydrolysis with Endo-F, a control experiment was included using a rabbit antiserum (6081) raised against a synthetic peptide with the amino acid sequence 1-14 of the 14K T3 fragment. The material used in these immunoblotting experiments contained mainly the 14K T3 δ -chain and little glycosylated 20K T3 δ -chain (Fig. 4, lane 1). As no 14K T3 polypeptide chain was recognized by the mono-

clonal reagent SP6, we concluded that this reagent was specific for the T3 ϵ -chain. The experiment with the rabbit antipeptide antibody (6081) and SP6 also demonstrated that the N-terminal sequence of the 14K T3 (T3 δ -chain) cannot be found in the other 20K T3 structures (T3 ϵ -chain).

We conclude that the T3 ϵ -chain differs from the T3 δ -chain in several respects. First, peptide mapping, N-terminal sequences and the use of antibody reagents showed that their primary structures are unique. Second, labelling with ^{125}I INA¹² and ^{125}I -TID demonstrated that the T3 ϵ -chain binds these hydrophobic reagents much more strongly than the other T3 chains. In fact, the ϵ -chain contained at least 10 times more ^{125}I -TID than did the T3- δ -chain (data not shown). The observation that both hydrophobic nitrene- and carbene-containing reagents preferentially label T3 ϵ -chain supports the notion that this molecule has an extensive hydrophobic area. An alternative

possibility, that the T3 ϵ -chain contains amino acids that are highly reactive with both the nitrene and carbene reagents, cannot be excluded. However, as anti-T3 and anti-T-cell receptor antibodies cause a rapid Ca^{2+} influx into the T cell, we have postulated previously that the T3 complex may contain a calcium channel²². The T3 ϵ -chain would be a good candidate for a constituent of such an ion channel.

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Cloning and expression of murine interleukin-1 cDNA in *Escherichia coli*

Peter T. Lomedico, Ueli Gubler,
Christopher P. Hellmann, Mitchell Dukovich*,
Judith G. Giri*, Yu-Ching E. Pan, Kenneth Collier,
Raina Semionow, Anne O. Chua & Steven B. Mizel*

Department of Molecular Genetics, Hoffmann-La Roche, Inc.,
Roche Research Center, Nutley, New Jersey 07110, USA

* Microbiology Program, The Pennsylvania State University,
University Park, Pennsylvania 16802, USA

Interleukin-1 (IL-1), a peptide hormone produced by activated macrophages, possesses the ability to modulate the proliferation, maturation and functional activation of a broad spectrum of cell types¹⁻⁷ and may play a major role in the initiation and amplification of immune and inflammatory responses through its action on these diverse cell populations⁸. IL-1 exhibits microheterogeneity in terms of its relative molecular mass (M_r , 13,000-19,000) and charge properties⁹, and although murine IL-1 has been purified^{9,10} and some of its basic structure-function relationships have been elucidated⁸, it has proved difficult to prepare sufficient amounts of IL-1 for direct and detailed sequence and structural studies. Here we report the cloning, sequence analysis and expression of murine IL-1 cDNA in *Escherichia coli*. The IL-1 cDNA codes for a polypeptide precursor of 270 amino acids. Biologically active IL-1 was produced in *E. coli* by expressing the carboxy-terminal 156 amino acids of the IL-1 precursor.

Recently, we reported the preparation of goat antibodies directed against murine IL-1¹⁰. Because of the specificity of these antibodies, we were able to develop an assay for the detection of IL-1 mRNA and analyse the primary translation form of IL-1 as well as the kinetics of its synthesis in activated macrophages¹¹. Poly(A)⁺ RNA was prepared from lysates of superinduced^{9,10} and uninduced P388D₁ cell line macrophages and translated in rabbit reticulocyte lysates. The translation

Table 1 Biological activity of recombinant IL-1

Preparation	μg Protein	$\Delta\text{c.p.m. } ^3\text{H-TdR}$ incorporation
Crude <i>E. coli</i> extract	0.7	26,370 $\pm$ 4,229
	1.4	57,637 $\pm$ 6,099
	2.8	63,940 $\pm$ 2,434
	7.0	93,475 $\pm$ 10,062
Purified recombinant IL-1	0.00002	9,997 $\pm$ 4,175
	0.00007	23,952 $\pm$ 3,733
	0.00014	46,561 $\pm$ 11,088
	0.00034	81,966 $\pm$ 2,087

E. coli containing pIL1 (1-156) were shifted to 42 °C for 3 h and then extracted with 7 M guanidine hydrochloride. An aliquot of the crude extract was diluted in phosphate-buffered saline and tested in the thymocyte proliferation assay¹⁰. ^3H -thymidine (TdR) incorporation in the presence of extracts of *E. coli* containing the expression plasmid with an insert in the opposite orientation was equivalent to background levels (2,100 c.p.m.). The crude extract was concentrated by ammonium sulphate precipitation and then chromatographed on a 2.5 $\times$ 85 cm Ultrogel AcA54 column. Fractions containing IL-1 activity were pooled and applied to an anti-IL-1 immunoadsorbent column¹⁰. The IL-1 was eluted with 4M KSCN and an aliquot was assayed for biological activity. On 12.5% SDS gels, the IL-1 migrated as a single band with M_r of 17,400.

products were immunoprecipitated with anti-IL-1 IgG and analysed on SDS gels (Fig. 1). The messenger RNA from superinduced cells programmed the synthesis of a 33,000 M_r polypeptide which was immunoprecipitated with the anti-IL-1 antibodies (Fig. 1, lane 1), but not by normal goat IgG (Fig. 1, lane 3). Although present in markedly lower levels, the 33,000 M_r product was also synthesized in reticulocyte lysates supplemented with mRNA from uninduced cells (Fig. 1, lane 2). Purified low- M_r IL-1 blocked the immunoprecipitation of the 33,000 M_r peptide (Fig. 1, compare lanes 5 and 6), a finding that demonstrates the strong antigenic relationship between the two polypeptides. In parallel studies on the kinetics of IL-1 synthesis and secretion¹¹, we have established that the 33,000 M_r polypeptide is indeed a precursor for the low- M_r form of murine

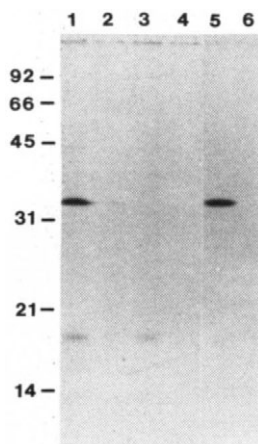


Fig. 1 Cell-free translation of P388D₁ IL-1 mRNA. Translation products were generated using rabbit reticulocyte lysates, immunoprecipitated with either normal goat IgG or anti-IL-1 IgG, and electrophoresed on SDS-polyacrylamide gels. Lanes 1 and 5, induced mRNA, translation products immunoprecipitated with anti-IL-1 IgG; lane 2, uninduced mRNA translation products immunoprecipitated with anti-IL-1 IgG; lane 3, induced mRNA, translation products immunoprecipitated with normal goat IgG; lane 4, uninduced mRNA, translation products immunoprecipitated with normal goat IgG; lane 6, induced mRNA, translation products immunoprecipitated with anti-IL-1 IgG in the presence of 2.5 μ g of purified IL-1.

Methods: P388D₁ cells were incubated with 10^{-8} M mezerein and $10 \mu\text{g ml}^{-1}$ cycloheximide for 4 h^{9,10}. Poly(A)⁺ RNA was isolated from these superinduced cells as well as from uninduced P388D₁ cells using the guanidine thiocyanate-CsCl method²³ and oligo(dT)-cellulose chromatography²⁴. Each translation reaction contained 1 μ g mRNA (1 μ l), 20 μ l rabbit reticulocyte lysate (Amersham, N.90), and 4 μ l ³⁵S-methionine (Amersham SJ 204). The reaction mixtures were incubated for 1 h at 30 °C and then treated with 400 μ g ml⁻¹ RNase and 20 mM EDTA for an additional 15 min. All subsequent manipulations were performed in the cold. Immunoprecipitation buffer (0.1 ml containing 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.35 M NaCl, 10 mM EDTA, and 25 mM Tris-HCl, pH 7.4) was added to each tube and the samples were 'precleared' with *Staphylococcus aureus*²⁵ (Pansorbin, Calbiochem) before overnight incubation with either normal goat IgG or anti-IL-1 IgG¹⁰. Immune complexes were precipitated using Pansorbin, extracted with 8 M urea, 1% SDS, 0.1 M dithiothreitol, 20 mM EDTA, and 50 mM Tris-HCl (pH 6.8) at 100 °C, and electrophoresed on 15% SDS-polyacrylamide gels. The gels were soaked in Enhance, dried and then exposed to X-ray film at -80 °C. *M_r* markers (shown $\times 10^{-3}$) were run in a parallel lane and visualized by Coomassie blue staining. In some experiments, a 18,000 *M_r* translation product was immunoprecipitated with normal and immune IgG. As purified IL-1 did not block the immunoprecipitation of this product, it represents nonspecific binding to goat IgG.

IL-1 which is normally isolated from the conditioned media of stimulated normal and cell line macrophages.

To enrich for the 33,000 *M_r* IL-1 mRNA, poly(A)⁺ RNA from induced P388D₁ cells was fractionated by centrifugation on a sucrose density gradient and analysed by cell-free translation (Fig. 2a). In this gradient, the mRNA coding for the 33,000 *M_r* IL-1 precursor exhibits a *M_r* slightly greater than *E. coli* 16S ribosomal RNA. Using this sucrose gradient enriched mRNA, a cDNA library of ~75,000 clones was constructed in pBR322 by established procedures¹². To identify an IL-1 cDNA clone, the library was randomly screened using the positive selection/translation analysis described by Parnes *et al.*¹³. After examining 150 pools (each containing 10 clones), a positive group was identified. One clone from this pool, termed pIL1 1301, specifically hybridized to IL-1 mRNA (Fig. 2b).

Clone pIL1 1301 contains a poly(A)⁺ tract and an insert of 1,701 base pairs (bp) (Fig. 3a). When used as a probe in a Northern blot against mRNA from induced P388D₁ cells, clone

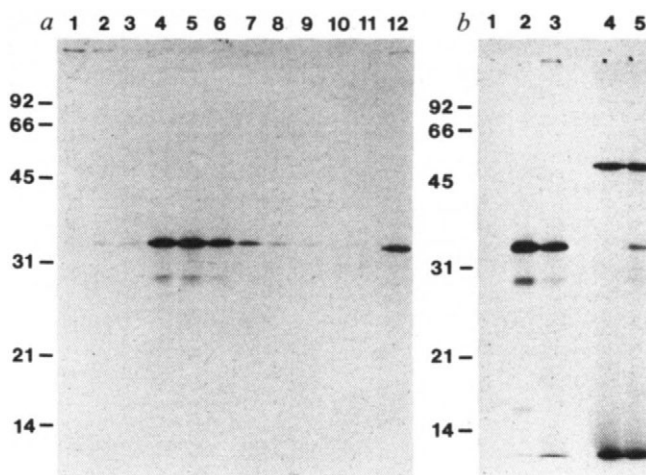


Fig. 2 Cloning of murine IL-1 cDNA. *a*, Translation and immunoprecipitation of polypeptides programmed by sucrose gradient fractionated P388D₁ mRNA. Lanes 1-11, 1 μ g of each sucrose gradient fraction; lane 12, 1 μ g of total P388D₁ poly(A)⁺ RNA. In a parallel gradient, *E. coli* 16S rRNA sedimented in the same fraction as the RNA sample used in lane 4, and 23S rRNA in the same fraction as the RNA sample used in lane 8. *b*, Translation and immunoprecipitation of polypeptides programmed by mRNA selected with clone pIL1 1301. Lanes 1 and 4, pBR322 selected mRNA; lanes 2 and 5, pIL1 1301 selected mRNA; lane 3, 1 μ g of total P388D₁ poly(A)⁺ RNA. In lanes 4 and 5 a portion of the total translation products were run directly on the gel, whereas in lanes 1-3, the translation products were immunoprecipitated with anti-IL-1 IgG before electrophoresis.

Methods: *a*, Superinduced P388D₁ poly(A)⁺ RNA (0.7 mg) was fractionated on a 39-ml 5-25% sucrose gradient by centrifugation for 19 h at 28,000 r.p.m. in a SW28 rotor. Twenty gradient fractions were collected and the RNA was recovered by ethanol precipitation. The RNA from each of the 11 fractions comprising the middle portion of the gradient was examined for IL-1 programming activity in the *in vitro* translation assay (1 μ g per reaction mixture). *b*, The fraction most highly enriched for the IL-1 mRNA (*a*, lane 5) was used to construct a cDNA library which was screened by hybrid-selection and cell-free translation as described by Parnes *et al.*¹³. After screening 1,500 clones (150 pools of 10), the candidate clone pIL1 1301 was compared with pBR322 in this assay. Plasmid DNA from 25 ml of bacterial cells was immobilized on nitrocellulose filters and hybridized to 20 μ g of unfractionated induced P388D₁ mRNA. The specifically hybridized mRNA was eluted and translated as described in Fig. 1 legend. The 49,000 *M_r* product in lanes 4 and 5 is synthesized in the reticulocyte system in the absence of added RNA.

pIL1 1301 specifically hybridized to a single 2,000 nucleotide mRNA species (Fig. 4). A restriction fragment near the 5' end of the pIL1 1301 insert was used in a primer extension experiment to demonstrate that the majority of the IL-1 mRNA molecules possess a single 5' terminus, of which ~270 nucleotides are missing from clone 1301 (data not shown). To define the missing sequences, a specifically primed clone bank was screened to identify a clone, termed pIL1 31, which overlaps with clone 1301 (Fig. 3b) and might contain a complete copy of the 5' end of the mRNA. The nucleotide sequence (Fig. 3a) of clones 1301 and 31 predicts a single open reading frame, starting at the first AUG from the 5' end, coding for a protein of 270 amino acids. This is likely to be the complete IL-1 precursor, as there is an in-frame termination codon upstream from the putative initiation codon. Hence, murine IL-1 mRNA is ~1,974 nucleotides long (excluding the poly(A) tail), codes for a protein of 270 amino acids, and possesses a 5'-noncoding region of approximately 60 nucleotides and a 3'-noncoding region of 1,104 nucleotides (Fig. 3b). We have sequenced several additional IL-1 cDNA clones to verify the sequence presented in Fig. 3. Colony hybridizations against the oligo (dT)-primed library indicate that 1 in 2,000 clones contain IL-1 sequences. Assuming a 10-fold enrichment following sucrose density cen-

a

AAG TCT CCA GGG CAG AGA GGG AGT CAA CTC ATT GGC GCT TGA GTC GGC AAA GAA	54
ATC AAG ATG GCC AAA GTT CCT GAC TTG TTT GAA GAC CTA AAG AAC TGT TAC AGT	108
MET Ala Lys Val Pro Asp Leu Phe Glu Asp Leu Lys Asn Cys Tyr Ser	10
GAA AAC GAA GAC TAC AGT TCT GGC ATT GAC CAT CTC TCT CTG AAT CAG AAA TCC	162
Glu Asn Glu Asp Tyr Ser Ser Ala Ile Asp His Leu Ser Leu Asn Gln Lys Ser	20 30
TTC TAT GAT GCA ABC TAT GGC TCA CTT CAT GAG ACT TGC ACA GAT CAG TTT GTA	216
Phe Tyr Asp Ala Ser Tyr Gln Ser Leu His Glu Thr Cys Thr Asp Gln Phe Val	40 50
TCT CTG AGA ACC TCT GAA ACG TCA AAG ATG TCC AAC TTC ACC TTC AAG GAG AGC	270
Ser Leu Arg Thr Ser Glu Thr Ser Lys MET Ser Asn Phe Thr Phe Lys Glu Ser	60 70
CGG GTG ACA GTA TCA GCA ACG TCA AGC AAC GGG AAG ATT CTG AAG AAG AGA CGG	324
Arg Val Thr Val Ser Ala Thr Ser Ser Asn Gly Lys Ile Leu Lys Lys Arg Arg	80
CTG AGT TTC AGT GAG ACC TTC ACT GAA GAT GAC CTG CAG TCC ATA ACC CAT GAT	378
Leu Ser Phe Ser Glu Thr Phe Thr Glu Asp Asp Leu Gln Ser Ile Thr His Asp	90 100
CTG GAA GAG ACC ATC CAA CCC AGA TCA GCA CCT TAC ACC TAC CAG AGT GAT TTG	432
Leu Glu Glu Thr Ile Gln Pro Arg Ser Ala Pro Tyr Thr Tyr Gln Ser Asp Leu	110 120
AGA TAC AAA CTG ATG AAG CTC GTC AGG CAG AAG TTT GTC ATG AAT GAT TCC CTC	486
Arg Tyr Lys Leu MET Lys Leu Val Arg Gln Lys Phe Val MET Asn Asp Ser Leu	130 140
AAC CAA ACT ATA TAT CAG GAT GTG GAC AAA CAC TAT CTC AGC ACC ACT TGG TTA	540
Asn Gln Thr Ile Tyr Gln Asp Val Asp Lys His Tyr Leu Ser Thr Thr Trp Leu	150 160
AAT GAC CTG CAA CAG GAA GTA AAA TTT GAC ATG TAT GCC TAC TCG TCG GGA GGA	594
Asn Asp Leu Gln Gln Glu Val Lys Phe Asp MET Tyr Ala Tyr Ser Ser Gly Gly	170
GAC GAC TCT AAA TAT CCT GTT ACT CTA AAA ATC TCA GAT TCA CAA CTG TTC GTG	648
Asp Asp Ser Lys Tyr Pro Val Thr Leu Lys Ile Ser Asp Ser Gln Leu Phe Val	180 190
AGC GCT CAA GGA GAA GAC CAG CCC GTG TTG CTG AAG GAG TTG CCA GAA ACA CCA	702
Ser Ala Gln Gly Glu Asp Gln Pro Val Leu Leu Lys Gln Leu Pro Glu Thr Pro	200 210
AAA CTC ATC ACA GGT AGT GAG ACC GAC CTC ATT TTC TTC TGG AAA AGT ATC AAC	756
Lys Leu Ile Thr Gly Ser Glu Thr Asp Leu Ile Phe Phe Trp Lys Ser Ile Asn	220 230
TCT AAG AAC TAC TTC ACA TCA GCT GCT TAT CCA GAG CTG TTT ATT GCC ACC AAA	810
Ser Lys Asn Tyr Phe Thr Ser Ala Ala Tyr Pro Glu Leu Phe Ile Ala Thr Lys	240 250
GAA CAA AGT CGG GTG CAC CTG GCA CGG GGA CTG CCC TCT ATG ACA GAC TTC CAG	864
Glu Gln Ser Arg Val His Leu Ala Arg Gly Leu Pro Ser MET Thr Asp Phe Gln	260
ATA TCA TAA AAG CAG CCT TAT TTC GGG AGT CTA TTC ACT TGG GAA GTG CTG ACA	918
Ile Ser	270
GTG TGT ATG TAC CAT GTA CAG GAA CCT TCC TCA CCC TGA GTC ACT TGC ACA GCA	972
TGT GCT GAG TCT CTG TAA TTC TAA ATG AAT GTT TAC CTT TGT AAG AGA AGA	1026
GCA AAC CCT AGT GGA GCC ACT CTG ACA TAT GAT ACT ATC TGT TAT TTT AFA GAG	1080
TAC CTT ATA GTT TGC TCA GTA CTA ATC ATT TTA ATT ACT ATT CTG CAT GGC ATT	1134
CTT AGS AGS ATT AAA AAG ACT CTA CAC ATA TTA CAG ATG GGT TAA CAA AGG GAT	1188
AAA ACA ACT GAA AAG CAC ACT CAA TGC ATT TGG AAT ATA AAT TCA CAG AGG AAT	1242
CTC ACT GTG CAC CTT CGG CTT CAA AAT GCC AGT TGA GTA GGA TAA AGG TAT AAG	1296
ACA TTA ATG CTG TCA TTT TCA AAA GGA AGS GGA CAA TAG CTA CAT CTT TCC TAC	1350
CTG AGT GGS TTT TAC TCC AGT GAG ATC ATT TGG ATG AAA TCC TCC TGT AAC AGA	1404
CCT CAA GAA GAG GAC GAG CTG TGG AAT GTT ATT TTT AAG TTA TTT TAT TAT TAT	1458
ATT TAT AAA TAT ATT TAT GAT AAT TAT ATT ATT TAT GGA ACA TCC TTA AAT CCT	1512
CTG AGC TTG ACA GGC ATC CTC ACA GCA GGA TTT TCT AAG TGG TCA GTT ABA TAT	1566
AGT TTC CTC TAG AGC ACC ATG CTA CAG ACT TTA CAC TTT TTC CAC AGC CAC GAA	1620
GCT CTC TGT ACA TTC CTG TAC TTG GGA GCC CTT TCA TCA TGA TCT TAA TCT GTA	1674
CTG TTT ACT TTG TCT ATC TAA AAT GAT AAT TGA GTC AGT CTT TTT CCC TCC CAT	1728
CCT TAA AGC TGT CTG GGT ATT CTT ACA TCA TTC AGT CTC ACC TGT AAC TAA CAC	1782
CAA CCA TCT TAA GAT GGA AAG AGC TTA ACT GTG ACA ACC ACA CTA CTG TTA CCT	1836
GAA GTT TCT TTT CTA GGT AAT CAG TGT TCT CCC TGG ATT CCA ATT TTT TTT	1890
TCA AAC CAC AGT ATC ATG TAA CTA TCA ACA ATA ACA ATC TCA TTA TTA TTA	1944
ATC ATA ATT AAA TAA AAC AAG TTT GAG CTG AAA AAA AAA AA	1974

2.9 —
1.5 —
1.3 —
1.1 —
0.9 —
0.6 —

Fig. 4 Northern blot analysis of IL-1 mRNA. Five μ g of induced poly(A)⁺ RNA was electrophoresed on a 1.4% glyoxal-agarose gel, transferred to nitrocellulose²⁷ and probed with nick-translated clone pIL1 1301. *M_r* markers (shown in kbp) were run in a parallel lane and visualized by ethidium bromide staining.

trifugation and equal cDNA cloning efficiencies, IL-1 mRNA accounts for 0.005% of the total poly(A)⁺ RNA from superinduced P388D₁ cells.

We have used two approaches to the mapping of the sequences responsible for IL-1 activity within the 270-amino acid precursor. Purified low-*M_r* P388D₁-derived IL-1 exhibits microheterogeneity on both Tris-glycinate and SDS-polyacrylamide gels^{10,14}. Attempts to obtain amino-terminal sequence data on this mixture were unsuccessful. However, when such IL-1 preparations were treated with cyanogen bromide, a number of peptides were released, of which two were isolated by means of HPLC in sufficient quantities for sequencing: peptide 1 $\rightarrow$ Lys-Leu-Val-Arg-Gln-Lys and peptide 2 $\rightarrow$ Ser-?-?-Tyr-Thr-Gln-?-Asp-Leu. Both of these sequences are found in the 170-amino acid precursor as deduced from the cDNA sequences (Fig. 3a). The sequence of peptide 1 is located at positions 130–135 and is preceded by a Met at position 129. The sequence of peptide 2 is located at positions 115–124, but is not preceded by a Met residue. We infer that peptide 2 is released by cyanogen bromide cleavage from the amino-terminal end of a subset of the IL-1 molecules that begin at the Ser residue at position 115.

We have also used expression in *E. coli* to prove that IL-1 is contained within the carboxy-terminal region of the 270-amino acid precursor. A 740-bp *Sau*3A fragment, beginning with the amino acid sequence of peptide 2 and containing the sequences coding for the last 156 amino acids of the 270-amino acid precursor, was isolated from pIL1 1301 and inserted into the *Bam*HI site downstream from an initiation codon in an *E. coli* expression plasmid (Fig. 5a). Expression in this plasmid (R. Crowl, manuscript in preparation) is controlled by a promoter regulated by a temperature-sensitive repressor. Guanidine hydrochloride (7 M) extracts of cells containing the recombinant plasmid were assayed for IL-1 activity in the murine thymocyte proliferation assay and for IL-1 immunoreactivity using goat anti-IL-1 IgG. Several clones containing the insert in the incorrect orientation made no detectable IL-1 activity or protein (Fig. 5b) at either 30 °C or 42 °C. Clones containing the insert in the correct orientation, pIL1 (1–156), made no IL-1 at 30 °C, but at 42 °C they synthesized a 17,400 *M_r* polypeptide that reacted with anti-IL-1 IgG (Fig. 5b) and was active in the thymocyte proliferation assay (Table 1). Crude extracts of pIL1 (1–156) contained 10⁷ units of IL-1 activity per gram of the recombinant clone. Using gel filtration chromatography and an anti-IL-1 immunoabsorbent, we have purified the recombinant IL-1 and have found that it possesses the same specific activity as the IL-1 released by P388D₁ cells ($\sim 6 \times 10^6$ units per mg, Table 1). The results of preliminary experiments indicate that the recombinant IL-1 possesses the same range of biological activities as the natural IL-1 produced by murine macrophages.

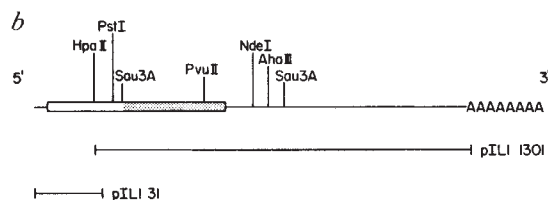
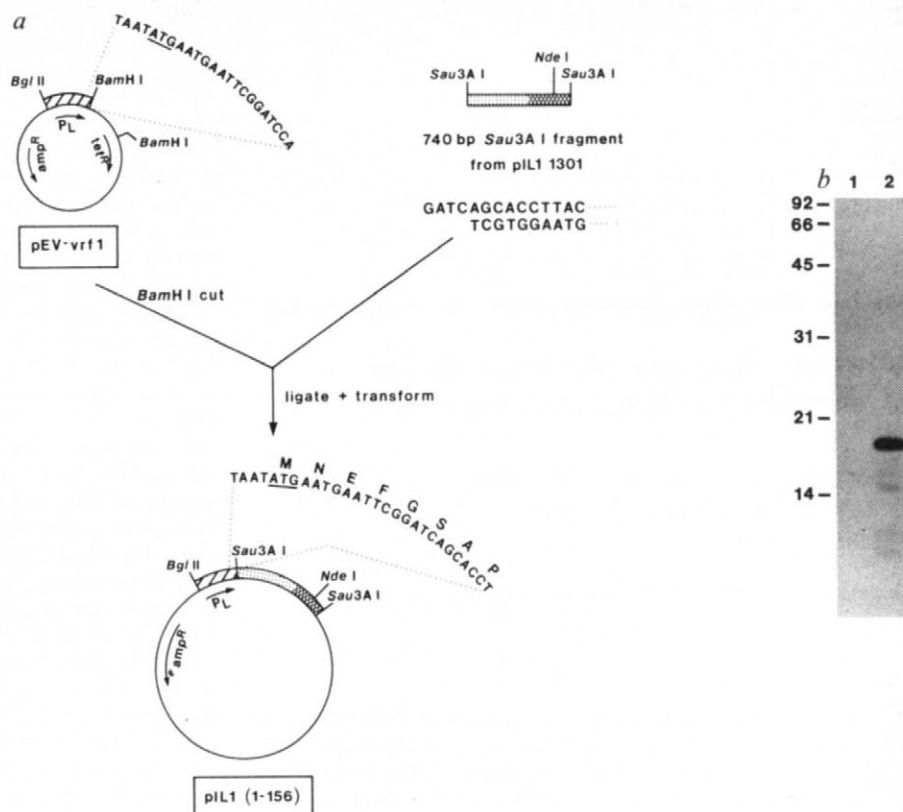


Fig. 3 Nucleotide sequence of IL-1 precursor cDNA and the predicted amino acid sequence of the IL-1 precursor. **a**, The nucleotide sequences of clones pIL1 1301 and pIL1 31 were determined by the chemical sequencing method²⁶. Nucleotide numbers are shown along the right-hand side of the sequence and the predicted amino acid sequence and corresponding position numbers are shown beneath. Clone pIL1 31 was isolated by using the oligonucleotide 5'-GTGAAGGTCTCACTGAACTCAGCCG-3', the sequence of which is derived from clone 1301 and complementary to positions 322–347 of the IL-1 mRNA, to generate and screen a specifically-primed library from sucrose-gradient enriched P388D₁ mRNA. **b**, Diagram of mouse IL-1 mRNA shows that clone 31 extends from nucleotide 1–302 and that 1301 extends from nucleotides 269–1,974 plus poly(A) tract. The boxed area indicates the position of the coding region for the 270-amino acid precursor and the stippled region indicates that portion containing IL-1 activity. For orientation purposes, certain unique restriction enzyme cleavage sites are indicated. The *Sau*3A sites are not unique but are included to indicate the position of the 740-bp fragment that was used for *E. coli* expression.

Fig. 5 Expression of IL-1 in *E. coli*. **a**, Expression clone pIL1 (1-156) was constructed by inserting a 740-bp *Sau*3A fragment from plasmid pIL1 1301 into the *Bam*HI site of pEV-vrf1 (ref. 15) and transforming *E. coli* strain RR1 containing the compatible plasmid pRK248 clts (ref. 28). Orientation of the insert in each clone was determined by the location of the *Nde*I site in the 3'-noncoding region of the IL-1 fragment. As indicated by the sequence around the initiation codon of the expression plasmid, this construction adds five additional amino acids to the amino-terminus of the expected 156 amino acid IL-1 polypeptide. **b**, *E. coli* containing the expression plasmid with an insert in the opposite orientation (lane 1) and in the correct orientation (lane 2) were grown in M9 media lacking methionine at 30 °C. When A_{550} reached 0.3, the cultures were shifted to 42 °C for 75 min. Forty μ Ci of 35 S-methionine were added to 0.5 ml of each culture and incubated for an additional 5 min at 42 °C. The bacteria were pelleted, solubilized in 25 μ l of 7 M guanidine hydrochloride and resuspended in 0.5 ml of immunoprecipitation buffer. Immunoprecipitation and gel analysis were performed as described in Fig. 1 legend.



The sequence of the cloned cDNA (Fig. 3a) and its expression in *E. coli* offers, for the first time, an appreciation of the primary structure of IL-1 and its precursor. The 270-amino acid precursor has a calculated M_r of 31,026, which is reasonably close to the estimated size (33,000) of the cell-free translation product (as assessed by SDS-gel electrophoresis). The precursor contains two cysteine residues (positions 14 and 47), three potential *N*-glycosylation sites (positions 66, 141 and 145) and an unusual tetrabasic region, Lys-Lys-Arg-Arg (positions 85-88). Homology searches of the GenBank Nucleic Acid Database and the NBRF Protein Sequence Database demonstrate that the sequence of the mouse IL-1 gene and protein have not previously been described. The sequence of IL-1 was compared with that of several other lymphokines, interleukin-2¹⁵, interleukin-3¹⁶, γ -interferon¹⁷, and granulocyte-macrophage colony-stimulating factor¹⁸, and no sequence homologies were evident. There is some weak homology (~32%) between the first 100 amino acids at the amino-terminal end of the mouse IL-1 precursor and the carboxy-terminal 100 residues of human and baboon α_1 -antitrypsin¹⁹. However, there is no homology with antithrombin III and ovalbumin, the other two members of this gene family^{19,20}.

The 270-amino acid precursor does not seem to contain a classic signal peptide. Hydropathicity plots²¹ indicate that the precursor is mostly hydrophilic—there is no sizeable hydrophobic region, especially at the amino-terminus. In addition, *in vitro* translation experiments using rabbit reticulocyte lysates and dog pancreas microsomes have not revealed any processing of the 33,000 M_r IL-1 precursor (data not shown). Furthermore, we have detected¹¹ the 33,000 M_r form of IL-1 in the culture fluid of stimulated macrophages—a finding that raises the interesting possibility that the 33,000 M_r peptide may be the species of IL-1 secreted by activated cells.

The carboxy-terminal 156-amino acid polypeptide that is active in the thymocyte proliferation assay has a calculated M_r of 17,992. The absence of cysteine residues in the low- M_r IL-1 provides an explanation for the insensitivity of IL-1 to reduction and alkylation⁸. Chou-Fasman calculations²² on this polypeptide predict a structure with extensive β -sheet character and little α -helicity.

In conjunction with other recent studies on IL-1 synthesis¹¹, the cloning, sequence analysis and expression of IL-1 cDNA clearly demonstrates that IL-1 from normal as well as cell-line macrophages is initially synthesized as a 270-amino acid precursor. Increased IL-1 mRNA levels (Fig. 1) probably indicate that this precursor is expressed in higher levels when the macrophage is stimulated. In some as yet unknown fashion, the IL-1 precursor is converted to the low- M_r form normally detected in the culture fluid of activated macrophages. Although additional studies remain to be done, we favour the hypothesis that the 33,000 M_r precursor is secreted and then enzymatically converted to a lower- M_r form. We suggest that one or more proteases released by the macrophage may be responsible for cleaving at a primary processing site (perhaps the tetrabasic Lys-Lys-Arg-Arg at position 85-89). Subsequent proteolytic activity would generate 'ragged' amino-termini, thus providing an explanation for the M_r and charge microheterogeneity that is characteristic of IL-1.

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Mitogens increase phosphorylation of phosphoinositides in thymocytes

Michael V. Taylor, James C. Metcalfe,
T. Robin Hesketh, Gerry A. Smith
& John P. Moore

Department of Biochemistry, University of Cambridge,
Tennis Court Road, Cambridge CB2 1QW, UK

In many cell systems the interaction of ligands with their receptors causes rapid breakdown and resynthesis of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂)^{1–5}. Recent work has focused on the role of the degradation products of PtdIns(4,5)P₂ as intermediates in the activation of cell function and growth⁶: inositol trisphosphate (InsP₃) can release Ca²⁺ from intracellular stores^{7–9} and diacylglycerol is thought to activate protein kinase C^{6,10}. This enzyme is also activated by phorbol esters (for example, 12-O-tetradecanoyl phorbol 13-acetate, TPA)¹¹ and this is assumed to account for the pleiotropic effects of TPA on cell function and growth. Mouse thymocytes are not mitogenically stimulated by TPA alone, but it is a potent co-mitogen in combination with either concanavalin A (Con A) or A23187 (A. N. Corps and J.C.M., unpublished observations). Here we show that mitogenic concentrations of TPA, A23187 and Con A¹² each cause an increase in the net phosphorylation of phosphatidylinositol (PtdIns) to PtdIns(4,5)P₂ in mouse thymocytes. This is consistent with stimulation by the mitogens of the same phosphoinositide phosphorylations in intact cells as recently demonstrated for the isolated products of the *src* and *ros* viral oncogenes in a cell-free system^{13,14}.

When mouse thymocytes were labelled with myo-[2-³H]inositol¹⁵, the amounts of ³H-label in each of the phosphoinositides increased at an approximately constant rate for 12–14 h, after which there was no further increase in incorporation. We assume, therefore, that the phosphoinositides are at isotopic equilibrium with ³H-inositol after 14 h and that subsequent changes in the amounts of ³H-PtdIns, ³H-PtdIns(4)P (phosphatidylinositol 4-phosphate) and ³H-PtdIns(4,5)P₂ reflect changes in the concentrations of these lipids.

TPA (10 nM) and A23187 (100 nM) stimulated increases in PtdIns(4)P and PtdIns(4,5)P₂ within 5 min and the amounts of ³H-label in both polyphosphoinositides continued to rise for ~15 min (Fig. 1a, b). Con A (1 µg ml⁻¹) caused a small, but variable, decrease in PtdIns(4,5)P₂ which amounted to typically 10% after 1 min, followed by a variable increase of up to 25% above the initial level after 10 min. In most experiments there was no initial decrease in PtdIns(4)P after Con A addition, and the amount of PtdIns(4)P increased by 30–60% after 10 min (Fig. 1a, b). The increased steady-state levels of the polyphosphoinositides stimulated by each of the mitogens were similar; these levels may be regulated in part by feedback inhibition of the phosphoinositide kinases by the polyphosphoinositides^{13,14,16}. There was no change in the amount of PtdIns over the time course of these responses (see Fig. 1 legend), thus it is clear that either the phosphoinositide kinases that synthesize PtdIns(4,5)P₂ from PtdIns via PtdIns(4)P (refs 1, 17) were

activated or that breakdown of PtdIns(4,5)P₂ was inhibited. This phospholipid is catabolized by a phosphodiesterase to release InsP₃ and diacylglycerol, and by a phosphomonoesterase to release PtdIns(4)P and phosphate^{18–20}.

Figure 1c, d shows the effects of the mitogens on the breakdown of PtdIns(4,5)P₂ by phosphodiesterase: neither TPA nor A23187 had any effect (±10%) on the amounts of either InsP₃ or the other inositol phosphates within 15 min. However, there was an increase in the level of InsP₃ within 30 s of the addition of Con A (Fig. 1c) and later increases in inositol bisphosphate (InsP₂) and inositol phosphate (InsP)¹⁵. The levels of InsP₃ were increased approximately sixfold after 5–8 min, then declined slowly. The stimulated rates of InsP₃ release and breakdown to InsP₂ and InsP must therefore be similar, as the total amount of inositol phosphates continued to increase for at least 15 min (Fig. 1d). Furthermore, the total amount of ³H-labelled inositol phosphates at 15 min was approximately twice that of ³H-labelled PtdIns(4,5)P₂ before the addition of Con A. The comparison is only approximate because the inositol phosphates recovered underestimate the extent of PtdIns(4,5)P₂ breakdown (some InsP is degraded to inositol even in the presence of Li⁺), and the percentage recovery of the polyphosphoinositides¹⁸ may be lower than that of the inositol phosphates (>85%; ref 15). Nevertheless, it is clear that Con A must cause a large compensating stimulation of polyphosphoinositide synthesis to account for the amounts broken down to inositol phosphates at 20 min. Con A, TPA and A23187 all stimulate net polyphosphoinositide synthesis at a similar rate (Fig. 1a, b). However, the actual rate of PtdIns(4,5)P₂ synthesis stimulated by Con A is at least 10-fold greater when the coincident stimulation of breakdown to inositol phosphates is taken into account. The data clearly indicate that Con A, but neither TPA nor A23187, activates polyphosphoinositide phosphodiesterase. The increase in PtdIns(4)P and PtdIns(4,5)P₂ stimulated by each of the three mitogens must, therefore, be due to either activation of the phosphoinositide kinases or inhibition of the phosphomonoesterases.

Although neither A23187 nor TPA had any significant effect on the amount of InsP₃ in the cells, they had opposing effects on the steady-state increase in InsP₃ stimulated by Con A (Fig. 1c), which can be analysed into effects on polyphosphoinositide synthesis and breakdown. TPA potentiated the steady-state increase in InsP₃ stimulated by Con A, although the initial rates of InsP₃ production were similar with and without TPA (Fig. 1c). This effect on InsP₃ production is most simply explained by the increase in the levels of PtdIns(4)P and PtdIns(4,5)P₂ in response to TPA plus Con A (not shown) compared with Con A alone, and any effect of TPA on the activated polyphosphoinositide phosphodiesterase or the inositol phosphate phosphatases appears to be small. In contrast to TPA, A23187 inhibited InsP₃ production by Con A (Fig. 1c) and as net polyphosphoinositide synthesis is stimulated by A23187 (Fig. 1a, b), the reduction in inositol phosphate levels may be caused either by a Ca²⁺-mediated inhibition of the activated polyphosphoinositide phosphodiesterase, or by stimulation of the inositol phosphate phosphatases.

Further indication of the mechanisms by which the synthesis and breakdown of PtdIns(4,5)P₂ can be altered was obtained by examining the effects of sodium azide and cyclic nucleotide analogues. When thymocytes were incubated for 10 min with sodium azide (10 mM), their ATP content was reduced from 3 mM to 0.75 mM; this reduced the amounts of PtdIns(4)P and PtdIns(4,5)P₂ by 15–20%, but had a negligible effect on the inositol phosphates in unstimulated cells (Fig. 2). However, azide reduced the stimulation of net polyphosphoinositide synthesis by the mitogens (shown for Con A in Fig. 2a, b). Azide also reduced the steady-state level of InsP₃ in Con A-stimulated cells, but not the initial rate of InsP₃ release (Fig. 2c). These effects can be attributed to a reduction in the phosphorylation of the polyphosphoinositides and are qualitatively similar to those reported in slices of parotid gland treated with 2,4-dinitrophenol¹.

Fig. 1 Stimulation of polyphosphoinositide synthesis (*a, b*) and breakdown (*c, d*) by mitogens. Thymocytes from 4–6-week-old BALB/c mice were prepared in an inositol-free culture medium and labelled for 18 h with *myo*-[2-³H]inositol as described previously¹⁵. The cells were washed twice by centrifugation in Earle's balanced salts solution buffered with 10 mM HEPES (supplemented with 5 mM LiCl^{30,31} in experiments where inositol phosphates were assayed) and incubated ($2.5\text{--}5 \times 10^6$ cells in 0.5 ml per sample) for 15 min at 37 °C. In each experiment the data for the phosphoinositides were obtained from triplicate determinations with a standard deviation $\leq 10\%$ of the mean. The data points for the inositol phosphates are single determinations, but are typical of data obtained in at least three experiments. *a, b*, At zero time the following additions were made: 10 nM TPA ($\square$), 100 nM A23187 (Δ), $1 \mu\text{g ml}^{-1}$ Con A ($\bullet$), or salts solution ($\circ$). The reactions were stopped by adding 1.88 ml chloroform/methanol (1:2), the phosphoinositides were extracted in acidic conditions, deacylated with a methylamine reagent and the glycerophosphorylinositides separated by anion-exchange chromatography¹. The amounts of PtdIns(4,5)P₂ (*a*) and PtdIns(4)P (*b*) are expressed as percentages of the amounts in untreated cells (3,280 and 1,080 d.p.m. per 10^7 cells, respectively), which were constant during the experiment. There was no change in the amount of PtdIns (190,000 d.p.m. per 10^7 cells) in unstimulated or mitogen-treated cells (TPA, A23187, Con A: $100.6 \pm 4.4\%$ of control ($n=6$), 98.1% ($n=2$) and $100.9 \pm 1.4\%$ ($n=6$) respectively). The data are from typical experiments: the mean values ($\pm$ s.d.) for PtdIns(4,5)P₂ 10 min after treatment with TPA, A23187 and Con A were $135.6 \pm 22.5\%$ ($n=6$), 122.4% ($n=2$) and $105.7 \pm 18.2\%$ ($n=6$) respectively; those for PtdIns(4)P were $192.1 \pm 36.8\%$ ($n=6$), 204.4% ($n=2$) and $139.3 \pm 16.1\%$ ($n=6$) respectively. *c, d*, Polyphosphoinositide breakdown to inositol phosphates. 10 nM TPA ($\square$, $\blacksquare$), 100 nM A23187 (Δ , $\blacktriangle$) or salts solution ($\circ$, $\bullet$) were added (open arrow) and the cells incubated for 10 min before addition of $1 \mu\text{g ml}^{-1}$ Con A to some aliquots of the cells as indicated by the closed symbols. The inositol phosphates were assayed as described elsewhere^{15,32,33} and the amounts of InsP₃ (*a*) and the sum of InsP, InsP₂ and InsP₃ (*b*) are expressed as percentages of the amounts in control cells (1,170, 180 and 360 d.p.m. per 10^7 cells, respectively). Note that A23187 (100 nM) had no effect on the ATP content of thymocytes (measured as in ref. 34) during the experiments shown.

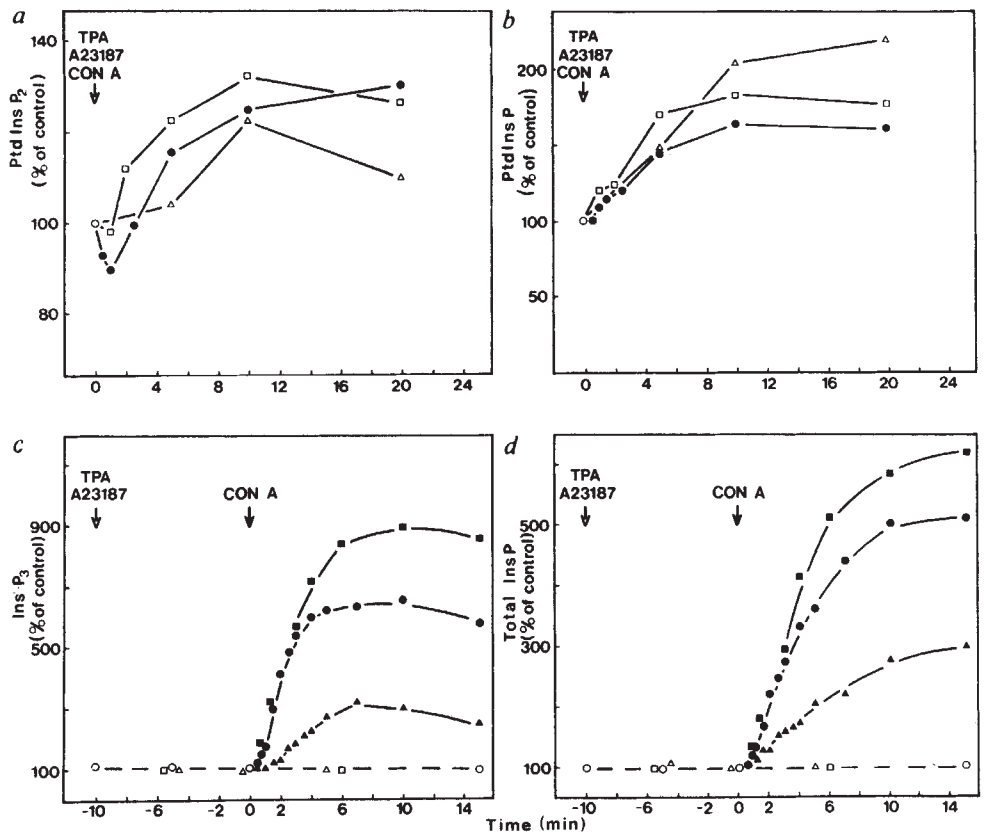
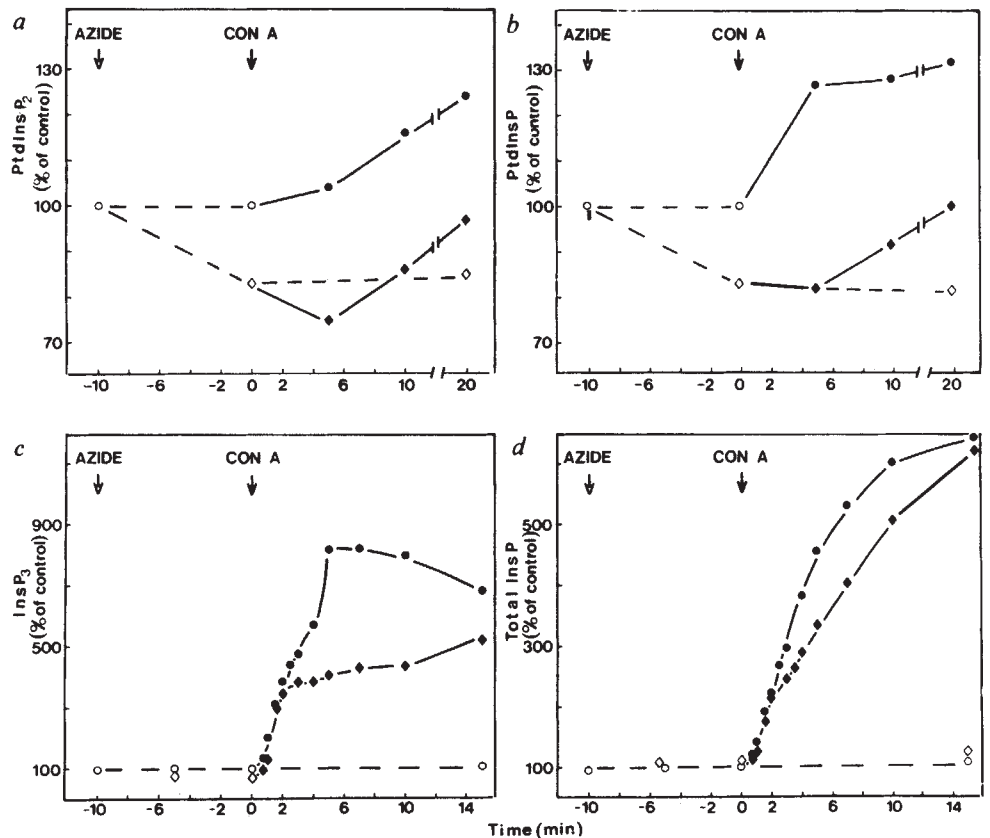


Fig. 2 The effect of azide on polyphosphoinositide synthesis (*a, b*) and breakdown (*c, d*). Experimental conditions were as described in Fig. 1 legend. Sodium azide (10 mM; $\diamond$, $\blacklozenge$) or salts solution ($\circ$, $\bullet$) was added at the times indicated by the open arrow and after 10 min Con A ($1 \mu\text{g ml}^{-1}$) was added to some aliquots of cells (closed symbols). *a, b*, Polyphosphoinositide data, average of two experiments. *c, d*, Production of InsP₃ and total inositol phosphates.



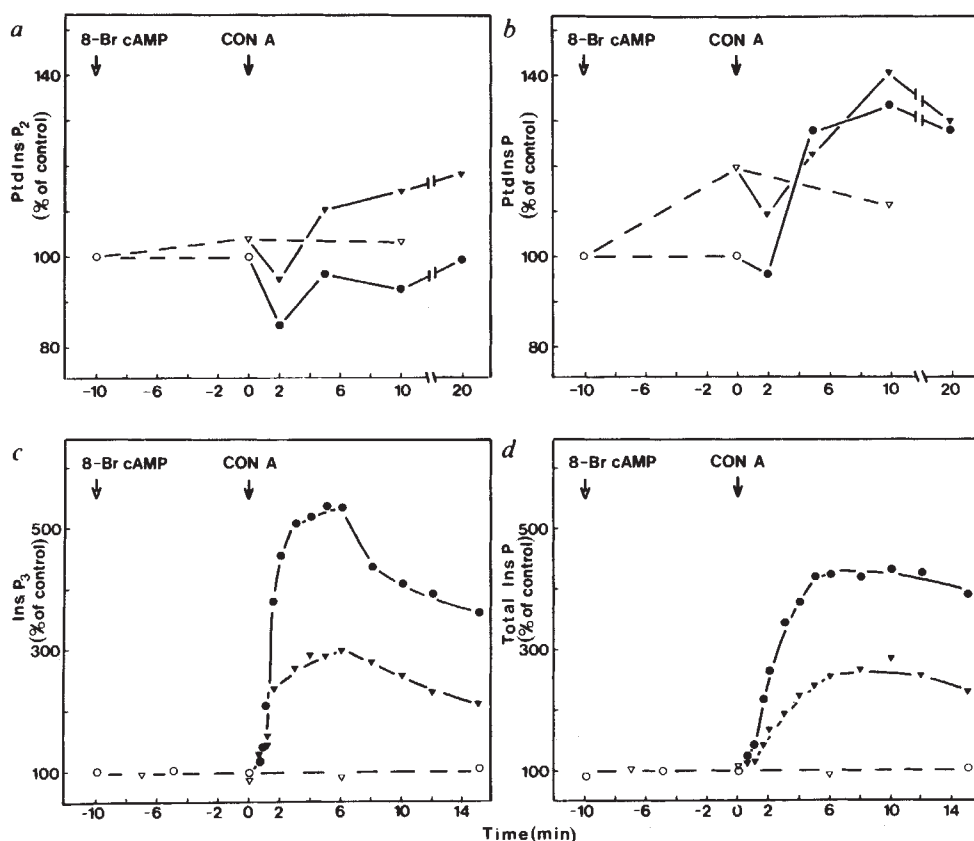


Fig. 3 The effect of 8-Br-cAMP on polyphosphoinositide synthesis (a, b) and breakdown (c, d). The experimental conditions were as described in Fig. 1 legend. 8-Br-cAMP (10 mM; ∇ , $\blacktriangledown$) or salts solution ($\circ$, $\bullet$) was added at the time indicated by the open arrow and after 10 min Con A ($1 \mu\text{g ml}^{-1}$) was added to some aliquots of cells (closed symbols). a, b, Polyphosphoinositide data, average of three experiments. 10 min after 8-Br-cAMP addition (no Con A), $\text{PtdIns}(4,5)\text{P}_2 = 103.8 \pm 5.5\%$ of control, $\text{PtdIns}(4)\text{P} = 119.7 \pm 6.2\%$ of control; in the same experiments, $\text{InsP}_3 = 105.0 \pm 7.2\%$. c, d, Production of InsP_3 and total inositol phosphates.

There have been several reports that cyclic nucleotides inhibit the phosphoinositide responses to ligands in a variety of cells²¹⁻²³. We have used 8-bromocyclic AMP (8-Br-cAMP), a membrane-permeant analogue of cyclic AMP, as thymocytes become desensitized to hormones which elevate cyclic AMP after prolonged incubation at 37 °C as required in these experiments (ref. 24 and J.P.M., unpublished observations). 8-Br-cAMP (10 mM for 10 min) increased the amount of $\text{PtdIns}(4)\text{P}$ by ~20%, but had no effect on $\text{PtdIns}(4,5)\text{P}_2$ (Fig. 3a, b). However, in cells stimulated with Con A, the amount of $\text{PtdIns}(4,5)\text{P}_2$ was increased in the presence of 8-Br-cAMP, although there was little effect on $\text{PtdIns}(4)\text{P}$. 8-Br-cAMP (but not 8-bromocyclic GMP; see ref. 23) inhibited production of inositol phosphates in response to Con A, although there was no effect ($\pm 10\%$) on inositol phosphate levels in the absence of Con A (Fig. 3c, d). The initial rate of InsP_3 production in response to Con A was not affected greatly by 8-Br-cAMP, but the steady-state level was reduced. These results suggest that 8-Br-cAMP has a small stimulatory effect on net polyphosphoinositide synthesis (as reported previously^{25,26}) and are also consistent with inhibition by 8-Br-cAMP of polyphosphoinositide phosphodiesterase after it has been activated by Con A. An alternative possibility is that 8-Br-cAMP activates the inositol phosphate phosphatases. The effects of 8-Br-cAMP on Con A-stimulated InsP_3 production are therefore similar to those of A23187, but their mechanisms of inhibition may differ because there are kinetic differences in their effects (see Figs 1c, d, 3c, d).

It is apparent from the experiments described here that each mitogen tested increases net polyphosphoinositide synthesis; whether this is mediated by stimulation of the phosphoinositide kinases or by inhibition of the phosphomonoesterases is unresolved. However, regulation of these enzymes clearly can occur via Ca^{2+} -dependent (A23187) or Ca^{2+} -independent (TPA) mechanisms, as TPA does not raise the intracellular free Ca^{2+} concentration in thymocytes²⁷. Con A stimulates the production of InsP_3 , which may be involved in generating the rise in Ca^{2+} in thymocytes in response to Con A^{27,28}, and the Ca signal may in turn stimulate polyphosphoinositide synthesis. Con A maintains prolonged production of inositol phosphates by an

approximate balance in the stimulation of both the synthesis and breakdown of $\text{PtdIns}(4,5)\text{P}_2$. Con A also causes the production of diacylglycerol²⁹, which may activate protein kinase C and hence stimulate net polyphosphoinositide synthesis by analogy with the effect of TPA. It is possible, therefore, that both products of $\text{PtdIns}(4,5)\text{P}_2$ breakdown act as feedback activators of its synthesis. It should, however, be emphasized that there is at present no direct proof that diacylglycerol produced by $\text{PtdIns}(4,5)\text{P}_2$ breakdown activates protein kinase C in intact cells, or that the small (less than threefold) rise in the Con A-induced intracellular Ca^{2+} concentration is sufficient to stimulate polyphosphoinositide synthesis. It is important to establish whether either of these mechanisms is operating, as they may occur in a wide range of cells. What is of interest, irrespective of mechanism, is that each of the mitogens stimulates the net phosphorylation of the phosphoinositides in lymphocytes. This finding may be compared with the recent observation that viruses carrying the *src* (ref. 13) and *ros* (ref. 14) oncogenes stimulate phosphoinositide phosphorylation in fibroblasts, which can be attributed to the phosphoinositide kinase activity of the isolated gene products.

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Two steroid 21-hydroxylase genes are located in the murine *S* region

Perrin C. White*†, David D. Chaplin‡, John H. Weis‡, Bo Dupont*, Maria I. New† & J. G. Seidman‡

* Laboratory of Human Immunogenetics, Memorial Sloan-Kettering Cancer Center, New York, New York 10021, USA

† Division of Pediatric Endocrinology, Department of Pediatrics, Cornell University Medical Center, New York, New York 10021, USA

‡ Department of Genetics, Harvard Medical School, Boston, Massachusetts 02115, USA

A common inherited disorder of steroidogenesis in man, 21-hydroxylase (21-OH) deficiency, is linked to the *HLA* major histocompatibility complex (MHC)¹, and is associated in particular with certain allotypes of the *HLA*-linked complement proteins^{2,3}. Recently, this disorder was demonstrated to result from a defective structural gene for the 21-OH enzyme, also termed cytochrome P-450_{C21} (ref. 4). The human (*HLA*) and murine (*H-2*) MHCs are homologous in overall organization and in the structures of their component genes⁵. To determine whether 21-OH genes are located in the *H-2* complex, we have now used a bovine adrenal complementary DNA clone encoding part of 21-OH⁶ to examine a cluster of overlapping cosmid clones derived from the *S* region of the BALB/c mouse⁷. We found that there are two 21-OH genes in this region, located immediately 3' to the *C4* and *Slp* genes.

The cDNA clone used in this study, pC21a, contains a 520-base pair (bp) insert in the *Pst*I site of pBR322, encoding approximately the middle third of the bovine 21-OH peptide⁶. To ascertain whether mouse genomic DNA contained a homologue of bovine 21-OH, the 430-bp *Pst*I fragment of pC21a was hybridized to Southern blots⁸ of BALB/c DNA (Fig. 1a). An *Eco*RI fragment of 3.0 kilobases (kb) and a 3.7-3.8-kb *Bam*HI fragment hybridized with the probe. In some experiments, the *Bam*HI band was resolved as a doublet.

Mapping of the chromosomal location of these genes was guided by linkage data obtained in man. Patients carrying 21-OH deficiency on the haplotype *HLA-Bw47; DR7* also carry a null allele at one of the two *C4* loci encoding the fourth component of complement in man^{2,3}. This haplotype invariably carries a deletion of a 21-OH structural gene, suggesting that the 21-OH and *C4* loci are adjacent and have both been deleted in this haplotype⁴. In man, both the *C4A* and *C4B* loci encode

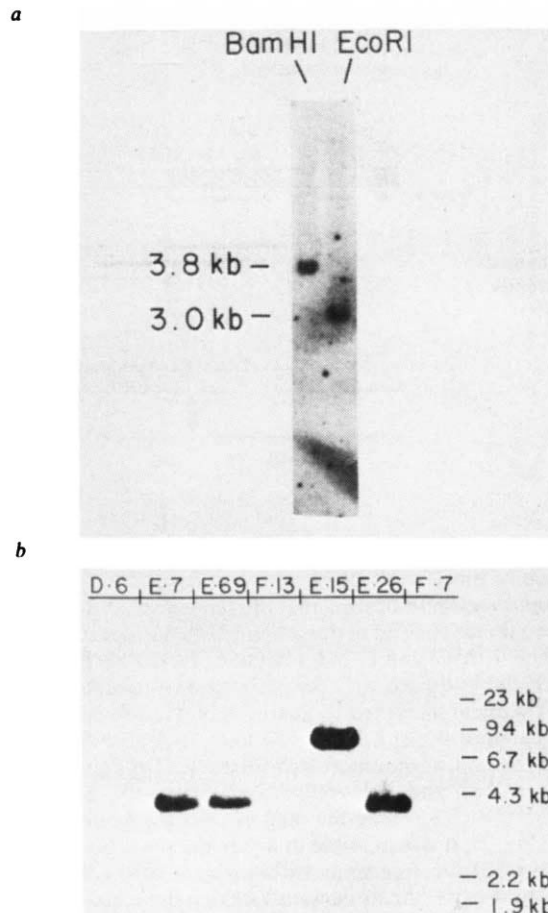


Fig. 1 Hybridization of pC21a to BALB/c mouse genomic DNA (a) and cosmid clones (b). **Methods:** a, 10 µg of DNA prepared¹⁹ from mouse liver were digested overnight with 4 U µg⁻¹ of the indicated restriction enzyme. The digests were subjected to electrophoresis in 1.0% agarose and blotted to nitrocellulose⁸. The 430-bp *Pst*I fragment of pC21a⁶ was radioactively labelled with ³²P-dCTP to a specific activity of 10⁹ d.p.m. µg⁻¹ by nick-translation²⁰. The blot was hybridized for 36 h at 65°C with 10⁶ d.p.m. cm⁻² of the denatured radioactive probe in 100 µl cm⁻² of hybridization solution: 0.9 M NaCl, 0.09 M Na-citrate, pH 7, 10% dextran sulphate, 0.5% SDS, 100 µg ml⁻¹ denatured herring sperm DNA, 0.1% Ficoll 400, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin. The blot was washed twice for 10 min each at room temperature in 0.3 M NaCl, 0.03 M Na-citrate, 0.5% SDS, and three times for 1 h each at 65°C in 0.15 M NaCl, 0.015 M Na-citrate, 0.5% SDS. The blot was autoradiographed for 3 days on Kodak XAR film using an intensifying screen at -70°C. Sizes of hybridizing fragments were determined by comparing electrophoretic mobilities with a *Hind*III digest of bacteriophage λ DNA. b, 2 µg of each cosmid DNA (prepared as described elsewhere²¹) were digested with *Bam*HI, fractionated on a 1.0% agarose gel and transferred to nitrocellulose. Hybridization and washing were performed as in a except that the probe was used at 5 × 10⁴ d.p.m. cm⁻².

haemolytically active complement⁹. There are two related loci in the mouse, located in the *S* region of the *H-2* complex. One encodes mouse *C4*, and the other, *Slp*, encodes 'sex-limited protein', a haemolytically inactive homologue of *C4* expressed only in males of certain strains^{10,11}. To determine whether the mouse 21-OH gene was linked to the *C4* or *Slp* genes, Southern blots were prepared from overlapping cosmid clones spanning the *S* region⁷ and hybridized with the pC21a probe (Fig. 1b). The probe hybridized with a 3.8-kb *Bam*HI fragment in cosmids E-7 and E-69 and a 3.7-kb *Bam*HI fragment in cosmid E-26, corresponding to the 3.7-3.8-kb *Bam*HI fragments seen in Southern blots of total genomic DNA (Fig. 1a). An 8.5-kb *Bam*HI fragment was detected in cosmid E-15 which contained

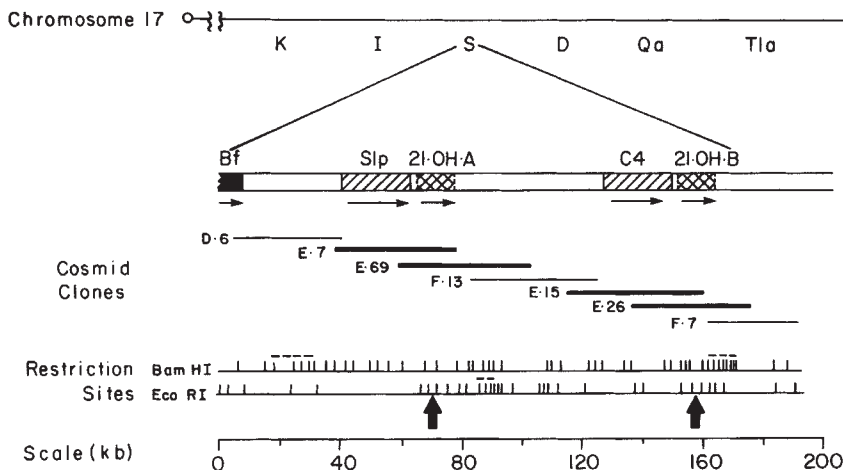


Fig. 2 Molecular map of the murine *S* region. The results of Fig. 1 are shown schematically. Cosmids⁷ carrying fragments which specifically hybridize with the pC21a probe are indicated by bold lines. Maps of *Bam*HI and *Eco*RI recognition sites are displayed above a scale in kb. A dotted line above restriction sites indicates that the order of fragments under the line has not been determined. The vertical arrows below the map indicate the fragments that hybridize to the pC21a probe. The proposed arrangement of the genes for factor B, *Slp*, *C4* and *21-OH* (*P-450_{C21}*) is illustrated at the top of the figure. Because the pC21a clone is not a full-length cDNA copy, the 5' and 3' borders of the two *21-OH* genes have not been determined and are therefore indicated by dotted lines. The direction of transcription is shown below each gene.

a portion of the 3.7-kb fragment seen in cosmid E-26 ligated to the cosmid vector, indicating that the sequence hybridizing with the probe was at one end of the genomic DNA insert. In addition, cosmids E-7, E-69 and E-26 all contained a 3.0-kb *Eco*RI fragment which hybridized with the probe and co-migrated with the *Eco*RI fragment identified in Southern blots of mouse genomic DNA (data not shown). In cosmid E-15, a smaller *Eco*RI fragment at the end of the insert hybridized with the probe.

Cosmids E-7 and E-15 respectively carry the *Slp* and *C4* genes^{7,12,13}. By inspecting the map of restriction sites for these clones (Fig. 2), it was possible to locate the sequences hybridizing with pC21a to fragments within 6 kb 3' of the *Slp* and *C4* genes. These experiments demonstrate that there are two *21-OH* genes in the mouse (which we have designated *21-OH-A* and *21-OH-B*) and that, as is true for at least one *21-OH* gene in man, they are tightly linked to the class III MHC gene, that for complement *C4*.

The adrenal cortex is the primary site of *21-OH* activity. To verify that the *C4*-linked *21-OH* genes were appropriately expressed in the mouse, the 3.8- and 3.7-kb *Bam*HI fragments from cosmids E-7 and E-26 were used to probe Northern blots of total RNA prepared from liver, testes and adrenal glands of C57BL/6 mice¹⁴ (Fig. 3). These probes hybridized to a 2.2-kb RNA species in total mouse adrenal RNA; this corresponds well to the size of *21-OH* mRNA isolated from bovine adrenal glands⁶. There was no apparent hybridization with unfractionated RNA from liver or testes, but very low levels of hybridization would not have been detected.

The orientations of the *21-OH* genes were determined relative to the *C4* and *Slp* genes. The 3.8-kb *Bam*HI fragment of cosmid E-7 (*21-OH-A*) was digested with *Kpn*I, yielding 2.7- and 1.1-kb subfragments. Restriction mapping demonstrated that the 2.7-kb fragment was closer to the 3' end of the *Slp* gene, whereas the 1.1-kb fragment was closer to the 5' end of the *C4* gene. The 1.1-kb *Bam*HI-*Kpn*I fragment was subcloned into the single-stranded bacteriophage vectors M13 mp8 and mp9 (ref. 15), and complementary single-stranded ³²P-labelled probes were prepared from each subclone¹⁶. The probe prepared from the mp8 subclone, but not the probe from the mp9 subclone, hybridized to a 2.2-kb species in total adrenal RNA (data not shown). Similar results were obtained with the 1.1-kb *Bam*HI-*Kpn*I fragment derived from the *21-OH-B* gene. These results demonstrate that both *21-OH* genes are transcribed in the same direction as the *Slp* and *C4* genes.

Previous study of these cosmid clones using several single-copy probes derived from regions flanking the *C4* and *Slp* genes⁷ indicated that the two genes arose by a direct duplication of about 55 kb of chromosomal DNA. The *21-OH* genes are located within this duplicated region; thus, single adjacent *C4* and *21-OH* genes were presumably duplicated simultaneously. The *Slp* gene product subsequently diverged from *C4* in function

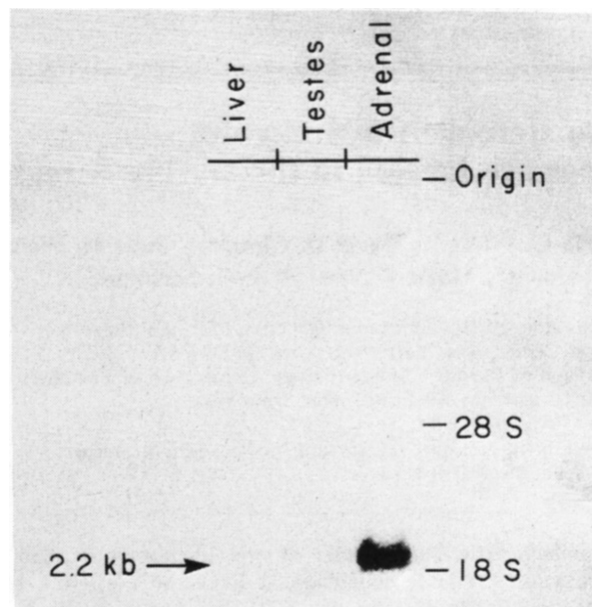


Fig. 3 Northern blot analysis using a murine genomic *21-OH* probe. Total RNA was isolated from the liver, testes and adrenal glands of C57BL/6 mice¹⁶. 15 µg of each RNA were fractionated by electrophoresis in denaturing conditions in 0.9% agarose-formaldehyde²² and transferred to nitrocellulose. The 3.8-kb *Bam*HI fragment of cosmid E-7 was nick-translated as described for Fig. 1a, and hybridized with the blot at 1×10^5 d.p.m. cm⁻² in 0.1 ml cm⁻² of: 50% formamide, 5 mM Tris-HCl pH 7.6, 0.75 M NaCl, 0.075 M Na-citrate, 12.5% dextran sulphate, 0.1% SDS, 0.1% Ficoll 400, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin, 0.1 mg ml⁻¹ denatured herring sperm DNA. The blot was hybridized at 42 °C for 18 h, washed as described for Fig. 1a and autoradiographed for 4 h at -70 °C without an intensifying screen. Molecular sizes were estimated by comparison with 18S and 28S RNA, assuming sizes of 2,100 and 4,200 nucleotides, respectively. The same result was obtained when the 3.7-kb *Bam*HI fragment of cosmid E-26 was used as the probe.

and regulation; at present, it is not known whether both or only one of the murine *21-OH* genes is active. There is also no evidence for concerted regulation of the *21-OH* and *C4* or *Slp* genes, but the *21-OH* genes may indirectly influence expression of *Slp* by modulating the production of adrenal androgens¹⁷.

Our mapping of the murine *21-OH* loci within the *S* region suggests that the *21-OH* genes became linked to the MHC before mammalian speciation. The functional significance of this linkage is unclear; it is possible that the *21-OH* genes have remained in the MHC because of their close proximity to the *C4* genes. Alternatively, as all other genes in this region have roles in

immune function, it is possible that 21-OH exerts an immunomodulatory role by regulating glucocorticoid biosynthesis. There may also be non-immunological effects; inbred mice display mating preferences depending on H-2 type, and can discriminate urine odours from H-2 congenic mice in Y-maze experiments¹⁸. H-2-associated differences in 21-OH activity would be expected to influence urinary levels of metabolites of both glucocorticoids and androgens, which might be perceived by mice. Such an influence on mating preference might encourage an advantageous mixing of mouse populations. An analysis of the regulation of the two 21-OH genes in different inbred mouse strains may help to answer these questions.

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Correction of feline arylsulphatase B deficiency (mucopolysaccharidosis VI) by bone marrow transplantation

P. W. Gasper, M. A. Thrall, D. A. Wenger*, D. W. Macy, L. Ham*, R. E. Dornsife, K. McBiles, S. L. Quackenbush, M. L. Kesel, E. L. Gillette & E. A. Hoover

Departments of Pathology, Radiation Biology, and Clinical Sciences, College of Veterinary Medicine and Biological Sciences, Colorado State University, Fort Collins, Colorado 80523, USA

* Department of Pediatrics, University of Colorado Health Sciences Center, Denver, Colorado 80262, USA

Feline and human mucopolysaccharidosis VI (MPS VI or Maroteaux-Lamy syndrome) are inherited autosomal recessive deficiencies of lysosomal enzyme arylsulphatase B¹⁻⁶. Affected cats and children exhibit lesions caused by incompetent degradation, retinal atrophy and excessive urinary excretion of dermatan facial dysmorphism, corneal stromal opacities, leukocyte granulation, retinal atrophy and excessive urinary excretion of dermatan sulphate—and usually die before adulthood⁷⁻¹¹. Most attempts to

treat humans affected with MPS VI or other mucopolysaccharidoses have been ineffective or logistically prohibitive¹²⁻²¹, but allogeneic bone marrow transplantation (BMT) offers promise for cure of certain inborn errors of metabolism²²⁻²⁴. Engraftment of normal donor marrow may endow the enzyme-deficient recipient with a continuous source of enzyme-competent blood cells and tissue macrophages to facilitate degradation of stored substrate and to prevent genesis of further malformations. To test this hypothesis, we performed allogeneic BMT in a 2-year-old male Siamese cat with advanced MPS VI. Here we describe BMT-induced correction of this hereditary enzyme deficiency.

The MPS VI-affected 2-year-old male Siamese cat used in this experiment was the offspring of two known MPS VI heterozygotes³. A healthy female sibling with normal levels of arylsulphatase B activity was selected as marrow donor after it was found to be histocompatible with the affected cat on the basis of one and two-way mixed leukocyte nonreactivity—the sole criterion available for determination of feline histocompatibility (for methods see Fig. 1). Total-body irradiation was delivered to the affected recipient cat as a single 7.0-Gy dose at a rate of 2.5 Gy min⁻¹ with a 6-MeV linear accelerator after selective decontamination of its intestinal tract with oral neomycin and polymyxin B for 7 days. At 24 h post-irradiation, female sibling donor bone marrow cells (2 × 10⁸ per kg body weight) were injected into the jugular vein of the irradiated MPS VI-affected cat (see Fig. 1). For 40 days thereafter, the cat was housed in a laminar flow sterile isolator where it received sterile food and water and its temperature, hydration, and fluid intake and output were monitored daily. Parenteral fluids and antibiotics were given as indicated by a deficit in hydration status and rectal body temperature greater than 39.2°C, respectively. Blood leukocyte and platelet concentrations, volume of packed red cells, leukocyte arylsulphatase B activity, and urinary glycosaminoglycan (GAG) excretion were determined before and after BMT (for methods see Figs 1, 2). Cyclosporin (15 mg per kg orally) was administered daily from 19 to 104 days post-BMT to prevent graft-versus-host disease. Karyotypic analysis of cultured bone marrow cells was performed 183 days after BMT.

Irradiation-induced myelotoxicity was evident on day 1 and extended to day 12 after BMT (Fig. 1). Beginning 18 days after BMT, rapid reconstitution by donor-origin granulocytes, monocytes and thrombocytes occurred. An early indication of donor-origin engraftment was a marked decrease in Alder-Reilly body-bearing neutrophils, and appearance of Barr body-bearing (female) neutrophils. Lymphocytes remained below pretransplant levels during cyclosporin therapy (days 19 to 104) but returned to normal levels thereafter. Urinary dermatan sulphate excretion decreased 2.5-fold by day 1 and 14.6-fold by day 232 after BMT (Fig. 2). Leukocyte arylsulphatase B activity increased 30-fold by 232 days after BMT. Both urinary dermatan sulphate excretion and leukocyte arylsulphatase B activity have maintained values within the normal range. Karyotypic analysis on day 183 post-BMT revealed a stable chimaera of 72.5% donor-origin (female) and 27.5% recipient-origin (male) cells.

Clinical changes in the MPS VI-affected cat following restoration of arylsulphatase B activity by BMT have been complete resolution of corneal clouding and continuing resolution of facial dysmorphism (Fig. 3). Subjective changes in the cat after BMT include improved ability to walk and increased movement of the head, neck and mandible (for example, restored capacity to masticate dry food), increased suppleness of the haircoat and improved demeanour.

While the logic behind treating heritable disorders of bone marrow-derived cells with allogeneic BMT is evident (reviewed in ref. 23), the rationale for treating multisystemic lysosomal storage diseases, such as MPS VI, with allogeneic BMT is less clear. Implicit in the application of BMT therapy for conditions in which the biochemical defect is expressed in organs not derived from bone marrow, is the belief that either the adopted enzyme-replete cells will transfer enzyme to cells of the several involved organs²⁵⁻²⁸, or that bone marrow-derived cells are

Fig. 1 Peripheral blood cell numbers in the MPS VI-affected cat marrow transplanted with normal allogeneic feline bone marrow. Total-body irradiation-induced myelotoxicity (days 0–18) followed by bone marrow transplantation-mediated haematological reconstitution (days 18–25) and stabilization (days 25–250).

Methods: At the times indicated, complete blood counts and serum chemistry evaluations were performed. Ficoll-metrizoate (1.077 g cm^{-3})-separated leukocytes from each of four siblings of the MPS VI-affected cat were assayed for one and two-way mixed leukocyte reactivity against similarly separated lymphocytes of the affected cat according to a modification of Peck and Bach³⁶. Lymphocytes (2×10^5) were mixed in 96-well plates either with 2×10^5 mitomycin C-treated ($2 \mu\text{g}$ mitomycin C per 10^6 lymphocytes, 45 min incubation at 37°C) or with 2×10^5 non-mitomycin C-treated leukocytes and incubated in a 37°C , humidified, 5% CO_2 atmosphere for 7 days. Eighteen hours before collection, $0.5 \mu\text{Ci}$ of ^3H -thymidine was added to each well. The cat with the lowest stimulation index was selected as the marrow donor. Marrow was obtained from the ketamine-anaesthetized donor by aspiration from both femurs and humeri. The cells were diluted in Hank's balanced salt solution, centrifuged at $1,000g$ for 10 min, the surface fat layer was removed, and the buffy coat cells were separated by pipette aspiration and then filtered through six layers of sterile gauze. Viable mononuclear cells were enumerated with the aid of Trypan blue vital stain and 2×10^8 mononuclear cells per kg infused into the jugular vein. Following haematological reconstitution (day 21) cyclosporin (a gift from J. F. Borel, Sandoz) was administered orally, 15 mg per kg daily. Serum trough levels of cyclosporin were measured at $134 \mu\text{g l}^{-1}$ (University of Minnesota Hospitals, Outreach Program). Karyotypic analysis was performed as follows: aspirated bone marrow mononuclear cells from the affected cat were cultured for 4 days in 25 cm^2 flasks as described³⁷, medium containing nonadherent cells were transferred to a 15-ml conical centrifuge tube, incubated for 1 h at 37°C with $4 \mu\text{g}$ of colchicine, and the pelleted cells were exposed to 5 ml of hypotonic (0.075 M) KCl solution for 10 min at 37°C and fixed with 1 ml of methanol:acetic acid (1:3). The final cell suspension was dropped on cleaned glass slides and dried at 65°C , and stained with a 2% Giemsa stain for 6–8 min. Forty metaphase chromosome were examined at $\times 1,000$.

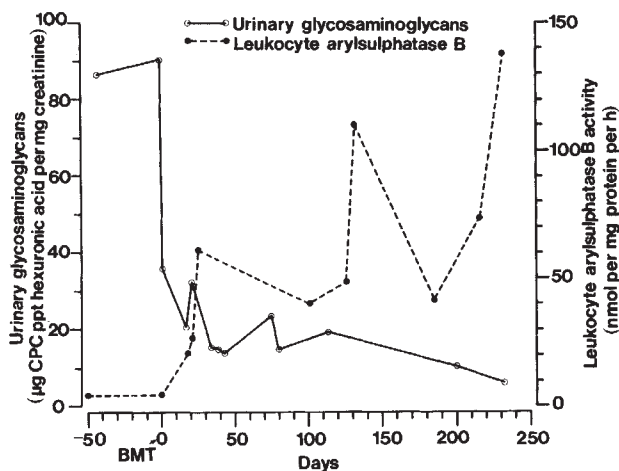
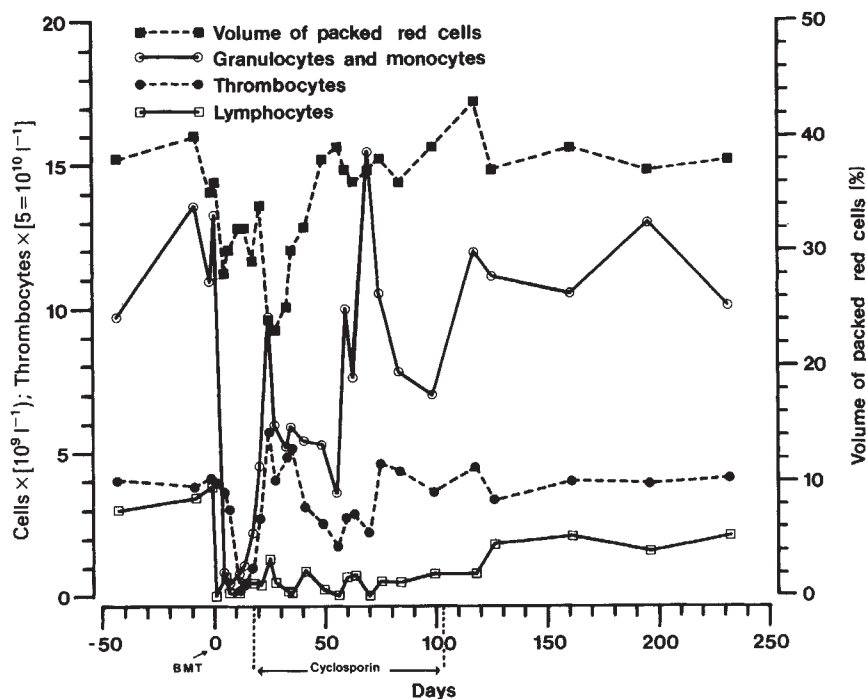


Fig. 2 Urinary excretion of glycosaminoglycans and leukocyte arylsulphatase B activity of the MPS VI-affected cat marrow transplanted with normal feline bone marrow. Urinary excretion of glycosaminoglycans decreased to the normal range (day +18 to day +232) as arylsulphatase B activity was installed in the mucopolysaccharidosis VI-affected cat by bone marrow transplantation. Urinary glycosaminoglycans were determined according to a modification of Pennock³⁸. CPC ppt, cetylpyridinium chloride precipitate. The predominant glycosaminoglycan excreted pre-BMT was dermatan sulphate, as determined by thin-layer chromatography. Leukocyte arylsulphatase B activity was determined according to a modification of Baum *et al.*³⁹.

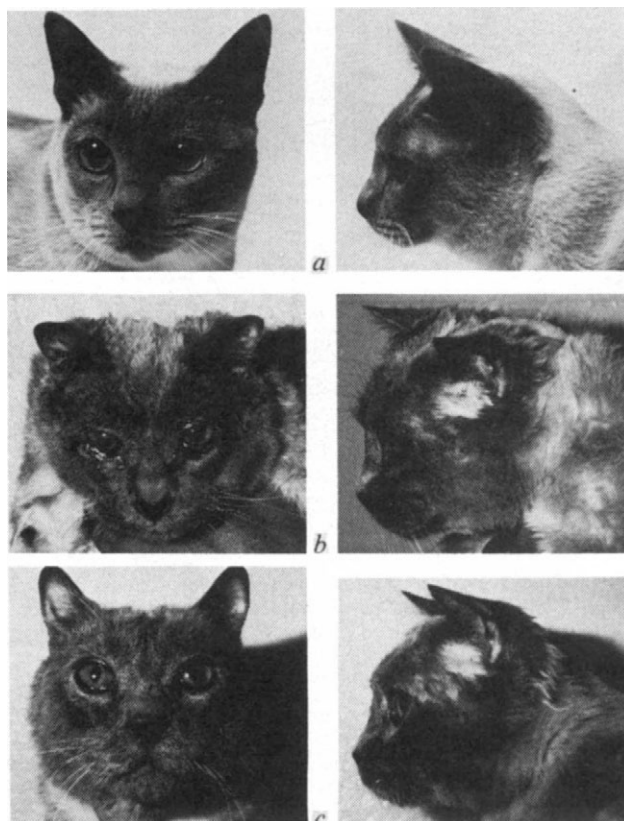


Fig. 3 a, Normal Siamese cat; b, arylsulphatase B-deficient sibling of the cat shown in a; c, the same animal 105 days after bone marrow transplantation. Complete resolution of corneal clouding and partial resolution of facial disfigurement can be seen.

responsible for degradation of the majority of systemic substrate under normal conditions. Support for one or both of these mechanisms is provided by the demonstrated correction of the galactosylceramidase deficit in the nerves of 'twitcher mice' when these nerves are grafted into normal mice²⁹.

Although allogeneic BMT has been performed in children with various lysosomal storage diseases, the long-term effectiveness of this treatment is unknown³⁰⁻³⁵. Our results show significant and sustained improvement in the health of an MPS VI-affected cat after successful allogeneic BMT, which suggests that allogeneic BMT may provide curative therapy for human MPS VI. However, in those lysosomal storage diseases where significant neurological damage occurs, this method must be further evaluated before it can be conscientiously recommended.

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Investigations using this animal model of human disease could clarify the pathogenesis of MPS-induced lesions and would allow detailed analysis of the ability of BMT to prevent and correct pathological changes caused by arylsulphatase B deficiency and perhaps other inborn enzyme deficiencies.

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Unusual abundance of vertebrate 3-phosphate dehydrogenase pseudogenes

M. Piechaczyk, J. M. Blanchard, S. Riaad-El Sabouty, C. Dani, L. Marty & P. Jeanteur*

Laboratoire de Biologie Moléculaire, Université des Sciences et Techniques du Languedoc, Place Eugène Bataillon, 34060 Montpellier Cedex, France and Centre Régional de Lutte contre le Cancer, Hôpital St Eloi, BP 5054, 34003 Montpellier Cedex, France

Only one gene coding for glyceraldehyde 3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12), a key enzyme in the control of glycolysis, is known to be functional in man, mouse, rat and chicken¹⁻⁶. The gene has been localized to chromosome 12 in human^{2,3} and chromosome 6 in mouse⁴. Only a single mRNA species has been found in chicken⁷⁻⁹ and rat¹⁰. However, analysis of genomic DNA blots of various species with a cloned GAPDH cDNA probe has revealed large differences in the level of reiteration, ranging from one to over 200 copies. On this basis, we have grouped these organisms into three classes according to the number of GAPDH-related sequences they contain; one class with a unique representation (chicken), another class of relatively low reiteration (10-30 copies in man, hare, guinea-pig and hamster) and a third class of high reiteration (>200 copies in mouse and rat). The third class represents the first reported occurrence of such an extreme number of pseudogenes related to an enzyme-coding gene and suggests that a dramatic amplification event took place between 15 and 25 million years ago.

As a preliminary to the screening of genomic libraries to obtain the gene, we probed Southern blots¹¹ of chicken and rat genomic DNA, respectively, with GAPDH cDNA clones from rat (pRGAPDH1)¹⁰ and chicken (pGPD1)⁷. In chicken DNA, we obtain a simple pattern of *EcoRI* (10 and 15 kilobases (kb)) and *HindIII* (8, 1.7 and 0.5 kb fragments) (Fig. 1). The *HindIII* pattern is identical with that observed by Kuo *et al.*¹² and the *EcoRI* pattern is also in good agreement with previous reports^{6,12}, despite the presence of a weaker additional band, probably reflecting some polymorphism at *EcoRI* sites. All these data support the existence of a single GAPDH sequence in the chicken genome. In contrast, however, Fig. 1 shows a large number of fragments homologous to GAPDH cDNA in the rat genome. This clone consists exclusively of coding sequences (spanning amino acids 261-324)¹⁰, so this hybridization pattern does not result from repetitive sequences in untranslated regions¹³. Moreover, the probe is so small as to make it extremely unlikely that the multiple bands would correspond to genomic fragments harbouring different exons. To eliminate the possibility that the pattern of multiple bands detected results from incomplete digestion of the genomic DNA, the same blots were rehybridized to a rat serum albumin cDNA clone, yielding a pattern similar to the one published for this gene¹⁴ (data not shown). The stringency of washing conditions (0.2×SSC, 60°C) suggests that these multiple genomic bands are highly homologous to GAPDH. Indeed, the same pattern was observed when filters were washed either at 25°C in 2×SSC or at 73°C in 0.2×SSC (not shown). This is therefore a clear indication for a highly multigenic family of related sequences in the rat genome. To rule out a preferential reiteration of only part of the GAPDH sequence as observed previously in the human myoglobin gene¹⁵, we rehybridized mouse and rat Southern blots with a *PstI*-*HinFI* fragment excised from a full-length rat cDNA clone. This probe is 229 nucleotides long, including 74 5' untranslated and 155 coding nucleotides; it gave rise to the same complex pattern of bands (not shown), indicating that these

* To whom correspondence should be addressed, at Hôpital St Eloi.

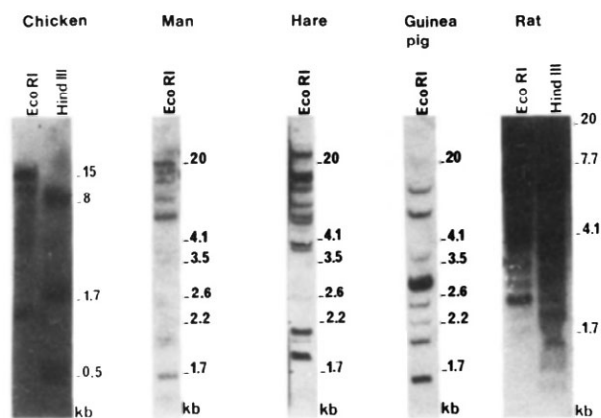


Fig. 1 Southern blot analysis of GAPDH-related sequences in chicken, hare, man, guinea pig and rat genomic DNA.

Methods: Genomic DNAs were prepared according to Gross-Bellard *et al.*²⁶. Hare DNA was a gift of Dr Y. Pauplin. Genomic DNAs (20 µg for chicken, man, guinea pig and hare, 5 µg for rat) were digested for 24 h with a 10-fold excess of the indicated restriction enzyme (Boehringer) and electrophoresed onto a 0.5% agarose gel (Sigma, type II) at 2 V cm⁻¹ for 20 h in Tris-borate-EDTA buffer (90 mM/90 mM/2 mM, pH 8.3). *EcoRI* and *HindIII* fragments of adenovirus 2 DNA were used as molecular weight markers (kilobases). Blotting onto nitrocellulose membranes was as described by Southern¹¹. Hybridizations were carried out in 50% formamide, 0.75 M NaCl, 50 mM sodium phosphate pH 7, 0.1% polyvinylpyrrolidone, 0.1% Ficoll, 0.5% SDS, 400 µg ml⁻¹ denatured sonicated salmon sperm DNA and 5–10 ng ml⁻¹ of nick-translated radioactive probe (1–2 × 10⁸ d.p.m. µg⁻¹, Amersham). The chicken blot was probed with a chicken cDNA clone (pGPD-1)⁷, the human, hare, guinea pig and rat blots with pRGAPDH-1 (ref. 9). Three 20 min washes in 2 × SSC (0.3 M NaCl, 0.03 M Na citrate) at room temperature were followed by a more stringent wash in 0.2 × SSC at 60 °C for 1 h. Autoradiography was performed at -80 °C on Kodak XAR5 film with an intensifying screen. Exposure times were 4 days for chicken, human, guinea pig and hare and 16 h for rat.

amplified sequences were not truncated significantly at the 5' end.

We next determined more accurately the number of sequences related to GAPDH cDNA and sought to determine when the amplification events occurred during evolution. Human, hare, guinea pig, hamster, mouse, chicken and rat DNA was analysed by Southern blot and dot-blot assay using a rat cDNA probe (pRGAPDH-1) (Fig. 2). The stringency of the washing conditions (0.2 × SSC, 60 °C) is such that only the least divergent sequences are scored, so that the values obtained are minimal estimates. We grouped the organisms into three classes according to the number of related sequences they contain. Class I has a single copy (for example chicken) and class II organisms have 10–30 copies (for example man, hare, guinea pig, hamster). The number of bands in Southern blots (Fig. 1) is consistent with the number of gene copies titrated by dot-blot assay (Fig. 2). The number of human GAPDH genes was confirmed by a homologous cDNA probe¹⁶. Class III (for example, rat, mouse) organisms contain an unusually high number of sequences (>200 copies), consistent with the results of screening a rat genomic DNA cosmid library with the pRGAPDH-1 probe, yielding an unexpectedly high number (140) of positive clones per ~0.8 genome equivalent (not shown), that is, approximately one clone per gene copy. To eliminate any possible artefact of the dot-blot assay, the same blot was probed successively with the rat serum albumin cDNA clone used above as a control of genomic blots and pRGAPDH-1. As expected, only one copy of albumin gene was titrated under these conditions (data not shown), confirming our finding of multiple copies of GAPDH-related sequences.

The high number of bands observed on all but chicken genomic blots (Fig. 1, hare and rat) argues against a clustering

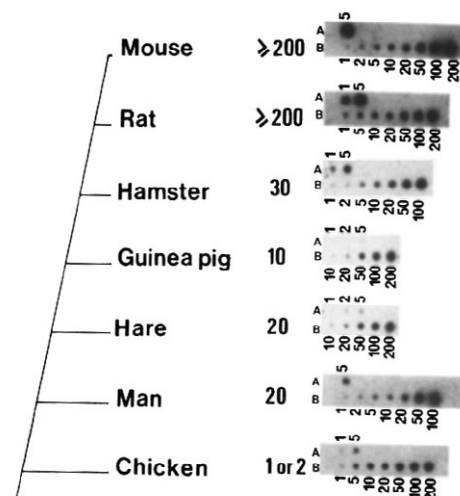


Fig. 2 Dot-blot quantitation of GAPDH-related sequences in various species. Lane A, indicated amounts (in µg) of denatured genomic DNAs probed with nick-translated chicken pGPD1 for chicken DNA and with pRGAPDH-1 for DNA from all other species. Lane B, scale of known amounts of pGPD1 or pRGAPDH-1 DNA 2 hybridized with the homologous probe in parallel with genomic DNA. The amount of plasmid DNA deposited on each spot of lane B was adjusted so that the numbers indicate directly the number of GAPDH sequences per 5 µg genomic DNA as spotted in A. The number of genes on the divergence tree are averages of independent experiments (5 for human, mouse, rat and hamster and 3 for chicken, hare and guinea pig). The probe used in all cases had comparable specific activities (1–1.5 × 10⁸ d.p.m. µg⁻¹). Samples of genomic and plasmid DNA were denatured by boiling for 10 min in 0.3 M NaOH at 50 µg ml⁻¹, chilled on ice and neutralized with one volume of 4 M ammonium acetate. Fixation onto nitrocellulose membranes used the BRL HybriDot system. Hybridization and washing were as described in Fig. 1.

of GAPDH-related sequences. At the chromosomal level, the presence of a processed gene on the human X chromosome^{17,18} indicates that these sequences are not confined to the human chromosome 12, the site of the only functional gene^{2,3}. At the level of rat genomic DNA, the rough equivalence between cosmid clones and gene copies mentioned above further indicates that there should not be more than one copy per ~40 kb.

The three classes of reiteration seem to have arisen through at least two steps of amplification which, according to the divergence tree of Fig. 2, could be dated back to more than 70–80 million years¹⁹ (after the separation of birds and mammals) and to 15–25 million years^{19,20} (after separation of Cricetidae and Muridae phyla but before that of species within the Muridae). However, the high degree of sequence conservation between rat and all other organisms except chicken argues against the first amplification step. Using a value of 5 × 10⁻⁹ nucleotide substitutions per site per year as a mutation rate for a non-functional gene²¹, one would expect at least 35% divergence between the true gene (represented by the cDNA probe) and pseudogene to have arisen before the separation of human from the other species analysed (70–80 million years). This is certainly not the case as, under the hybridization stringency used here, these pseudogenes would not be detected. Indeed, rat and chicken cDNA which have diverged by 19% (ref. 10) did not cross-hybridize under our stringency conditions.

The nature of the amplification processes is thus open to speculation. Duplications and random insertions of cDNA sequences (processed genes or retrogenes) in the genome are two mechanisms currently proposed for pseudogene generation. As mentioned above¹⁸, one such processed gene has been identified on human chromosome X, which indicates that random insertions can account for at least some of the GAPDH family.

In the case of human arginosuccinate synthetase, which shows approximately the same level of reiteration frequency (10–15 copies)²², it has been shown that most if not all are processed genes²³. Both GAPDH and arginosuccinate synthetase are ubiquitous housekeeping enzymes which are therefore likely to be expressed in germ-line cells where retrogenes are believed to be generated. Based on the above considerations, our hypothesis is that most, if not all, pseudogenes for these two enzymes could have arisen in man by insertion of cDNA copies. The same may also be true for the other members of the same class of reiteration (hare, guinea pig and hamster).

On the other hand, several considerations make it unlikely that the second mechanism alone could have produced such a remarkable multigenicity as observed in Muridae. First, insertion of cDNA copies in such a high number has never been observed, even for genes encoding other ubiquitous proteins such as actin²⁴ or dihydrofolate reductase²⁵ for which mRNA templates are available constantly. Second, a preliminary analysis of several genomic clones shows that different clones have slightly different patterns of stained bands but similar patterns of GAPDH-hybridizing bands, suggesting that duplication events involving DNA fragments of several kilobases in length occur in these organisms.

Our findings make it possible to speculate on the relative contribution of these two processes to the generation of the GAPDH family in Muridae. In a first step common to all species studied, except chicken, 10–30 pseudogenes would be contributed by insertion of cDNA copies. At this point, Muridae would have departed from other species by a burst of duplication events (possibly by transposition-like mechanisms) to reach the extreme degree of reiteration observed. A detailed analysis of our characterized rat genomic clones is now necessary to elucidate the mechanisms involved in this latter step.

During the completion of this work, we learned that A. Hanauer and J. L. Mandel (personal communication) have observed values quite similar to ours for the multigenicity of human, hamster and mouse.

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Intensification of the 5.9-nm actin layer line in contracting muscle

I. Matsubara, N. Yagi, H. Miura*,
M. Ozeki* & T. Izumi†

Department of Pharmacology, Tohoku University School of Medicine, Seiryō-machi, Sendai 980, Japan

* X-ray Technical Division, JEOL Ltd, Nakagami, Akishima, Tokyo 196, Japan

† Department of Engineering Physics, Chubu University, Matsumoto, Kasugai 487, Japan

According to the cross-bridge model^{1–3} of muscle contraction, an interaction of myosin heads with interdigitating actin filaments produces tension. Although X-ray equatorial diffraction patterns of active (contracting) muscle show that the heads are in the vicinity of the actin filaments, structural proof of actual attachment of heads to actin during contraction has been elusive⁴. We show here that during contraction of frog skeletal muscle, the 5.9-nm layer line arising from the genetic helix of actin⁵ is intensified by as much as 56% of the change which occurs when muscle enters rigor, using a two-dimensional X-ray detector. This provides strong structural evidence that myosin heads do in fact attach during contraction^{6,7}.

Two-dimensional detectors of multiwire type^{8–10}, despite their advantage over the one-dimensional detector, have not been applied to structural studies on muscle because the spatial resolution in the direction perpendicular to anode wires is limited. We have improved the resolution by the method¹¹ used in a wide-angle detector, that is by increasing the drift distance for electrons liberated on ionization. The improved detector, like other position-sensitive detectors, has the capability of fast recording and low background and is therefore suitable for studying transient changes of weak X-ray reflections such as arise from the actin filaments during contraction.

The basic structure of the detector was the same as that described previously^{12,13}. Anode wires were sandwiched between two planes consisting of cathode strips (Fig. 1). These strips were parallel to the anode wires in the back plane and orthogonal in the front plane. An avalanche occurring to an anode wire induced positive pulses on nearby strips on both planes. Such pulses were transmitted to delay lines^{8,14}. The *x* coordinate of the avalanche was determined from the signal propagation times to both ends of the delay line in the back plane, and the *y* coordinate from those in the front plane.

The detector had a sheet of aluminized Mylar with a strong negative bias near the entrance of the chamber. This enlarged the space for electrons to drift and multiply, increasing the number of anode wires involved in each avalanche. In consequence, the accuracy of the position determination along the *x* axis was improved considerably. With an optimum spacing (4 cm) between the Mylar and the anode, the spatial resolution (full width at half maximum) was 600 μm along the *x* axis and 500 μm along the *y* axis.

The outputs of the detector were sent to a buffer memory (2 × 10⁴ words) which stored them as a function of time. After a data-collection period, consisting of a resting phase before muscle contraction and an active phase during contraction, the contents of the buffer memory were transferred to a disk memory. The disk had separate memory segments for the two phases and accumulated the data on repetition of contractions to give rise to the diffraction patterns.

A sartorius muscle of the bullfrog *Rana catesbeiana* was placed in a low-angle camera of the mirror-monochromator type¹⁵ at a specimen-to-detector distance of 75 cm. The meridional and layer-line reflections at orders of 1/42.9 nm⁻¹, arising from myosin, were recorded up to the 6th order with a total exposure of 2–3 min. The exposure time needed for recording the much weaker actin layer line at 1/5.9 nm⁻¹ was 4–10 min, considerably shorter than the time (~24 h) required for

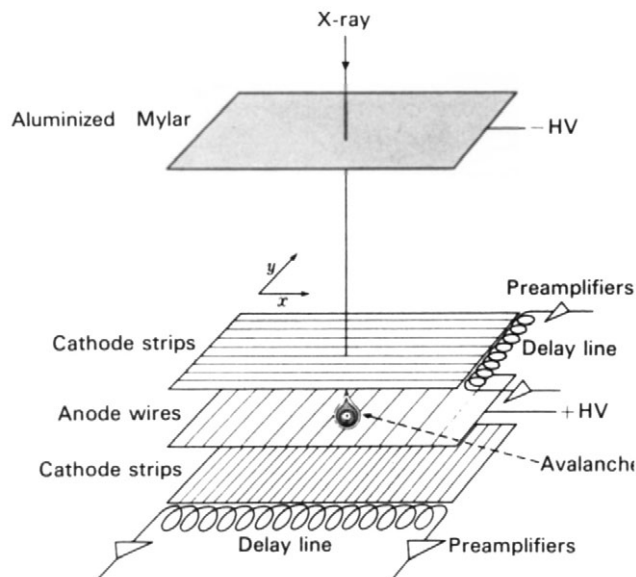


Fig. 1 Structure of the multiwire chamber. The chamber consisted of a sheet of aluminized Mylar (-1.0 kV) near the entrance, and an anode plane (80×80 mm, $+1.8$ kV) sandwiched by two cathode planes with a spacing of 3 mm. The anode plane was made of gold-plated tungsten wires $20 \mu\text{m}$ in diameter, 1.5 mm apart. The cathode planes were made of the same wires, 1 mm apart, orthogonal or parallel to the anode wires. The cathode wires were connected in pairs to form strips^{10,12}. The circuit for the delay-line readout was the same as that described by Hendricks¹³, containing four filter amplifiers, four discriminators and two time-to-digital converters. The sensitivity of the detector was not quite uniform along the x axis. When the uniformity was tested at a Mylar-to-anode distance of 4 cm with an uncollimated iron source, the photon count at each anode wire was 8% greater than that at the midpoint between wires. The chamber was filled with a gas mixture of 90% argon and 10% methane at a pressure of 1 atm. With a dead time of $7 \mu\text{s}$, the maximum counting rate was $4 \times 10^4 \text{ s}^{-1}$ with a 72% efficiency.

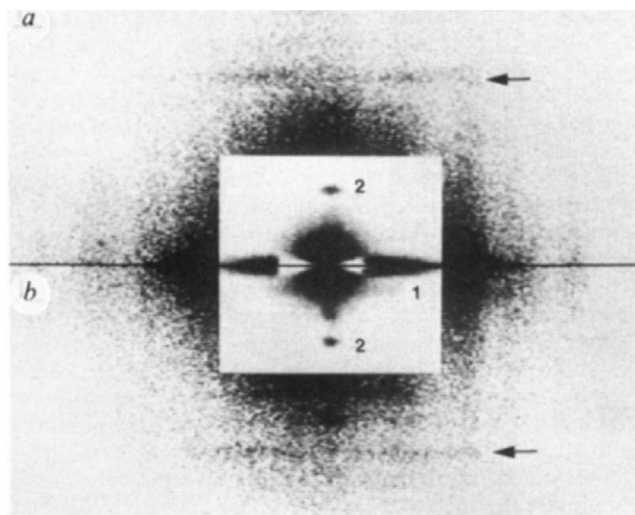


Fig. 2 Comparison of contracting (a) and resting diffraction (b) patterns. Arrows, actin layer lines at $1/5.9 \text{ nm}^{-1}$. In the central parts of the patterns, the contrast was adjusted to display the myosin layer line at $1/42.9 \text{ nm}^{-1}$ (1) and the meridional reflection at $1/14.3 \text{ nm}^{-1}$ (2).

Methods: A frog sartorius muscle was held in a specimen chamber by clamping the pubic bone at one end and connecting the tendon to a force transducer at the other end. The muscle axis was aligned parallel to the y axis of the detector. The specimen chamber was perfused with oxygenated Ringer's solution (115 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl_2 , 2.15 mM Na_2HPO_4 , 0.85 mM NaH_2PO_4 , pH 7.2) at 2°C . Isometric contractions of 1 s duration were elicited by trains of supramaximal pulses (20 Hz) given through a pair of platinum electrodes placed parallel to the muscle axis. Each muscle was made to contract 40 times, and the diffraction data from 20 muscles were accumulated to obtain the contracting pattern; the total exposure time was ~ 10 min. The average tension of the 40 contractions was $84 \pm 1\%$ ($\pm \text{s.e.m.}$, $n = 20$) of the initial tension. The X-ray source was a rotating-anode generator (Rigaku, type FR) with a fine focus (1×0.1 mm) on a copper target. This was operated at 50 kV with a tube current of 70 mA.

recording it on an X-ray film placed at the position of the detector.

We studied the change in intensity of this actin layer line on activation of muscles. Each muscle was stimulated to contract isometrically at a sarcomere length of $2.3 \mu\text{m}$ for 1 s every 5 min. The muscle produced a steady tetanic tension from about 0.15 s after the onset of the stimulus. The diffraction pattern obtained for a 0.8 s period during steady tension (Fig. 2a) was compared with the resting pattern (Fig. 2b) obtained for a 0.8 s period immediately before each contraction. During contraction, the integrated intensity of the actin layer line was $31 \pm 3\%$ ($\pm \text{s.e.m.}$, six pairs of patterns) greater than that during rest; the increase was statistically significant ($P < 0.01$). There was no apparent shift of the intensity peak along the layer line.

The layer line was intensified also in rigor muscles. The muscle was put into rigor while in the X-ray camera by first perfusing the specimen chamber (holding a living resting muscle at a sarcomere length of $2.3 \mu\text{m}$) with Ringer's solution containing 1 mM iodoacetate at 30°C for 1 h. The muscle then was stimulated repetitively with electrical pulses (1 Hz) until the resting tension increased and there was no response to stimulation. Finally the specimen chamber was perfused again with the iodoacetate Ringer for 1 h. In the rigor pattern the integrated intensity of the actin layer line was $55 \pm 7\%$ ($\pm \text{s.e.m.}$, five pairs of patterns) greater than that in the resting pattern. In addition, the radial position of the intensity peak was now closer to the meridian. Both the intensification of the layer line and the inward shift of the peak on rigor development had been shown previously¹⁵ and attributed to the attachment of myosin heads to actin.

When the behaviour of this layer line during contraction was

first studied using X-ray films^{15,16}, no change in the intensity was detected. Haselgrove¹⁷ did observe an intensity increase during contraction but, because of the large standard deviations of the measurements, he considered this result inconclusive. A one-dimensional position-sensitive detector revealed no intensity change¹⁸. The present study has demonstrated a significant intensity increase, more than 50% of that occurring on rigor induction. Dr H. E. Huxley (personal communication) and his co-workers have observed similar changes.

Other possible causes of an intensity increase of this layer line during contraction have been eliminated by Parry and Squire⁶, who concluded that the increase can be explained satisfactorily only in terms of the attachment of myosin heads to actin. Thus our result provides evidence for the attachment of cross-bridges in active muscles⁷, although at this stage we cannot rule out the possibility that other changes in the thin filament contribute to the increase. The absence of significant inward shift of the intensity peak along the layer line can be explained by assuming the attachment to occur at a variety of angles^{6,7}.

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A sequence downstream of AAUAAA is required for rabbit β -globin mRNA 3'-end formation

A. Gil & N. J. Proudfoot

Sir William Dunn School of Pathology, University of Oxford, Oxford OX1 3RE, UK

The sequence AAUAAA, found 11-30 base pairs (bp) upstream of the poly(A) site of most non-histone eukaryotic messenger RNAs (mRNAs)¹ forms an essential part of the recognition site for 3'-end processing of the primary transcript²⁻⁴. However, the sequence AATAAA is found in transcribed regions of genes⁵⁻⁸ and is differentially utilized in genes containing multiple copies of the sequence within the 3'-noncoding region⁸⁻¹⁸, suggesting that the hexanucleotide alone does not comprise a complete recognition site. Therefore, it seems likely that additional sequences are required to form a complete recognition site for 3'-end formation. We have investigated the sequence requirements for mRNA 3'-end formation using the rabbit β -globin gene as a model system. Here we demonstrate that an additional sequence 3' to AAUAAA is required for the correct 3'-end formation of rabbit β -globin mRNA.

To delineate possible sequence requirements for 3'-end formation within the 3'-flanking region of the rabbit β -globin gene, *Bal*31 deletions were made of sequences downstream to AATAAA (Fig. 1). A simian virus 40 (SV40)-pBR328 expression vector, p β 5'SV, containing the rabbit β -globin gene plus 425 bp of 5'- and 355 bp of 3'-flanking sequence¹⁹, was linearized at a unique *Sal*I restriction enzyme site 3' distal to the AATAAA and the ends digested with *Bal*31 exonuclease. This approach generated a series of clones with varying lengths of 3'-flanking deletions proceeding from a distal to proximal direction with respect to the AATAAA. A *Bgl*III fragment containing the 3'-noncoding region, the AATAAA plus the remaining 3'-flanking sequence was subcloned into the unique *Bgl*III site at the translation termination codon of the rabbit β -globin gene in the intact p β 5'SV plasmid; this provided cloning sites 5' and 3' to the insert containing the *Bal*31 deletion, ensured that sequences adjacent to the insert were identical between constructs, and also retained the original 3'-noncoding region, AATAAA and poly(A) site 3' to the insert as an internal control.

The DNAs from these deletion constructs were transiently expressed in HeLa cells²⁰; 36 h after transfection, the cytoplasmic RNA was extracted and analysed by S₁ nuclease mapping^{21,22} using an end-labelled probe spanning the entire region of the insert and the original 3'-flanking region downstream. RNAs from constructs showing correct 3'-end formation at the inserted site were poly(A)-selected (data not shown)²³.

Six constructs (clones 2-7) containing varying lengths of 3'-flanking deletions were analysed (Fig. 2). Clone 2, containing the largest 3'-flanking deletion ending 15 bp beyond the AATAAA, did not contain sufficient sequences to signal 3'-end

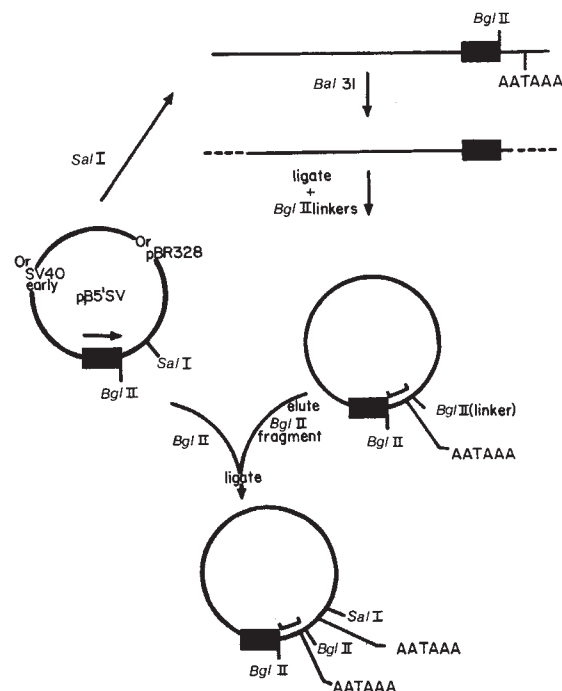


Fig. 1 Construction of *Bal*31 deletions 3' distal to the AATAAA sequence. The plasmid p β 5'SV containing the entire rabbit β -globin gene plus 425 bp of 5'- and 355 bp of 3'-flanking sequence was linearized with *Sal*I at a unique restriction enzyme site 3' distal to the AATAAA. Linearized DNA (100 ng) was digested with 0.1 U of *Bal*31 exonuclease in a total volume of 10 μ l at 30 °C for 35-40 min²⁷. Approximately 20 ng of this DNA were filled-in and the ends ligated in the presence of an equal weight of unphosphorylated *Bgl*III linkers. The ligated DNA was transformed into MC1061 competent cells. Ampicillin-resistant transformants were selected and the DNA from mini-preparations²⁷ was digested with *Bgl*III. The *Bgl*III fragment containing the 3'-noncoding region, AATAAA, plus the remaining 3'-flanking sequence was eluted from a 1% agarose gel²⁷. This fragment was subcloned into the unique *Bgl*III site at the translation termination codon in the rabbit β -globin gene of the intact p β 5'SV plasmid. The final constructs contained the insert flanked by *Bgl*III sites and the original 3'-noncoding region, AATAAA, and poly(A) site 3' to the insert.

formation within the inserted fragment. Instead, processing occurred 3' beyond, using the original AATAAA and poly(A) site (Fig. 3). The remaining five deletion constructs (clones 3-7) contained sufficient sequences to signal correct 3'-end formation 19 bp downstream of the inserted AATAAA. Of these five functional constructs, clone 3 contained the largest deletion of 3'-flanking sequence, ending 51 bp beyond the AATAAA, leaving a difference of only 35 bp between the end of this clone and the nonfunctional construct, clone 2. Within this 35-bp region is a G+T-rich homology, part of a pentanucleotide homology, and the poly(A) site. The G+T-rich homology, TGTGTTGGAA, is found 3' of the poly(A) site in several other genes²⁴. The 5' end of this critical 35-bp region intersects another sequence homology, CAYUG, which is found 3' of the AATAAA before or after the poly(A) site of many vertebrate genes²⁵. This pentanucleotide sequence is complementary to regions of the RNA of the U4 small nuclear ribonucleoprotein, suggesting that U4 may mediate 3'-end processing. The poly(A) site is found 19 bp 3' of the AATAAA and is therefore missing from the nonfunctional deletion clone 2. However, sequence analysis of many vertebrate genes has shown that all four nucleotides are used for polyadenylation (S. M. Berget, unpublished data), thus the exact sequence of the poly(A) site is unlikely to be important for mRNA 3'-end formation. It is more likely that either or both of the G+T-rich and pentanucleotide sequence homologies may

Fig. 2 Sequence 3' to AATAAA, indicating the ends of the *Bal31* deletion constructs, homologues found within the critical 35-bp region between the ends of the nonfunctional (2) and functional (3) clones, and the poly(A) site(*). The ends of the *Bal31* deletion clones 2 and 3 were determined precisely by DNA sequencing²⁸ while those of clones 4–7 were determined approximately by restriction enzyme mapping. The numbering system corresponds to that used in Fig. 3.

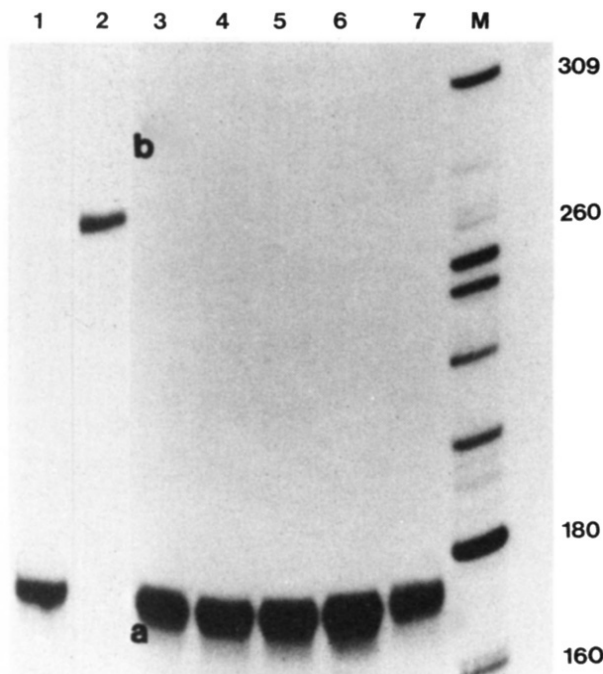
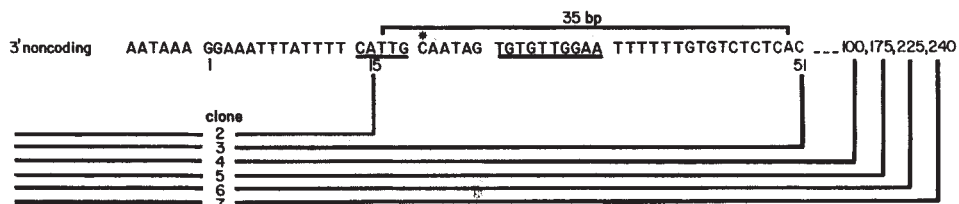


Fig. 3 S₁ nuclease mapping of the 3' ends of the cytoplasmic RNAs from *Bal31* deletion constructs of the rabbit β -globin gene. Lane 1, (control) RNA from intact rabbit β -globin gene in the p β 5SV plasmid showing correct 3'-end formation 19 bp 3' of the AATAAA. Lane 2, RNA from the nonfunctional deletion construct, clone 2, with insert ending 15 bp beyond the AATAAA; 3' ends were formed only at the original poly(A) site 3' to the inserted fragment. Lanes 3–7, RNAs from functional deletion constructs with inserts containing progressively greater lengths of 3'-flanking sequence. Clones are arranged in ascending order, starting with clone 3, which ends 51 bp 3' of the AATAAA. a, Correct 3'-end formation occurred in all five clones 19 bp downstream of the inserted AATAAA. b, Some 3' ends, not detected in clones 4–7, were formed at the original poly(A) site 3' of the insert in clone 3. Lane M, ³²P-labelled markers (bases). The numbering system corresponds to that used in Fig. 2.

Methods: 10 μ g of DNA from each deletion construct were transfected into a half-confluent 199-mm dish of HeLa cells by the calcium phosphate precipitation method²⁰. Five plates were used for each DNA; 12 h after transfection the medium was changed. The cells were grown for an additional 24 h, then the RNA was extracted and the cytoplasmic fraction purified²⁷. The rabbit β -globin RNAs were detected by S₁ nuclease mapping of the 3' ends²² using an end-labelled RI-Sal DNA probe spanning the region of the insert and the original 3'-noncoding region, AATAAA and 3'-flanking sequence.

be involved in the formation of a complete recognition site for 3'-end formation of the rabbit β -globin mRNA as the nonfunctional deletion clone 2 ends 15 bp beyond the AATAAA and lacks both of these sequences.

Aside from correct 3'-end formation at the inserted site, a low level of processing, not observed in clones 4–7, was detected at

the original poly(A) site 3' to the inserted site in clone 3 (Fig. 3). If the low level of 3'-end formation at the original site accurately measures the amount of readthrough RNA not processed at the inserted site, then there might be sequences 3' to the poly(A) site that are not required for correct 3'-end formation but nevertheless affect the efficiency of the process. Alternatively, there may be a transcription termination sequence within the immediate 3'-flanking region, resulting in less readthrough RNA processed at the original poly(A) site.

The experiments described here demonstrate that sequences within a critical 35-bp region extending from 3 nucleotides 5' to 31 nucleotides 3' of the poly(A) site are required for correct 3'-end formation of the rabbit β -globin mRNA. These results are consistent with the *Bal31* deletion analysis of the adenovirus E2A transcriptional unit recently described by McDevitt *et al.*²⁶; they demonstrated a sequence requirement for 3'-end processing downstream of the poly(A) site. However, the sequence of the required region of the E2A poly(A) site differs from that of the 35-bp region of the rabbit β -globin gene; this disparity may reflect the different factors involved in recognizing a signal sequence for 3'-end formation in a viral as opposed to a mammalian gene. As the functional clone ending 51 bp beyond the AATAAA includes the G+T-rich homology and the pentanucleotide homology, either or both of these sequences may be required for correct 3'-end formation. Further analysis by point mutation of the 35-bp region should elucidate the precise sequence requirement for 3'-end formation.

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Lines of investigation

J.T. Bonner

Scaling: Why is Animal Size so Important?

By Knut Schmidt-Nielsen.

Cambridge University Press: 1984. Pp. 241. Hbk £20, \$29.95; pbk £7.95, \$9.95.

Size, Function, and Life History.

By William A. Calder III.

Harvard University Press: 1984. Pp. 431. \$32.50, £26.

THERE has been a rash of interest recently in the ramifications of the effect of size on living organisms. Traditionally this is a subject that has been within the province of vertebrate physiologists, although it is now extending, with important messages, into the realm of ecology.

Professor Schmidt-Nielsen's *Scaling* is written for a general audience and is eminently readable; a splendid book primarily on the effect of animal size on physiology. Schmidt-Nielsen has himself done important work in the field and this has been one of his interests for many years. His latest account of the subject is clearly written in much the same easy-to-follow way that characterizes *Animal Physiology* (Cambridge University Press, 3rd Edn 1984), his well-known textbook. Professor Calder's book, on the other hand, is for the specialist. It covers some of the material on physiology that is in Schmidt-Nielsen, but goes on to look into the developmental and ecological consequences of size. This is a very difficult book to read, yet it is full of valuable, detailed information, well worth any trouble it might cause the reader in digging out the nuggets. It is more of a reference compendium than a book to read, but there are short portions scattered here and there that suddenly flash out as important and useful insights. When I finished reading these volumes I felt they could well have been glued together, with Schmidt-Nielsen first as a lucid introduction, followed by Calder with fascinating detail. Since this is not possible, I urge the unwary to read them in that sequence. The appropriateness of this is not entirely without cause for one gathers from Calder's preface that he is a student and a friend of Schmidt-Nielsen.

Both books begin with the idea that an allometric formulation is the most useful way to compare size-connected or size-invariant properties of different organisms. This method dates back at least 50 years, to the work of Julian Huxley who compared the growth rates of two developing structures in such a way that they produced straight lines when plotted logarithmically. The slope of the line reflects the change of proportions with absolute size increase. This same method can apply equally well to any measurement and its relation to body size, and is the way in which these two authors most frequently use their allometry. So, for instance, to give

the celebrated example of M. Kleiber (and others), if metabolism is determined for different-sized organisms, then

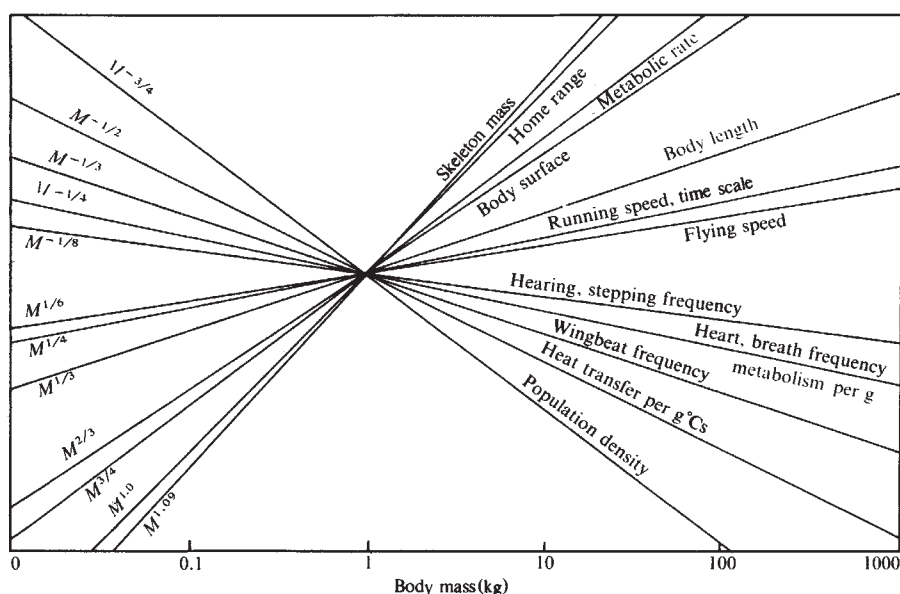
$$\text{metabolism} \propto \text{weight}^b$$

where, in this case, $b = 3/4$. By substituting other components for metabolism one can conveniently express how they change with weight. One could substitute heart rate, or gestation period, or speed of locomotion, or brain size, or clutch size, or life span, or population density or the size of the home range, for they all vary with the body size of the animal.

The diverse properties which are described in this way can be roughly grouped into the following three categories: physiological (or functional), developmental and ecological. Only Calder discusses the last two. On the importance of body size in physiology there is now a vast array of new data. For example, it is possible to show that even though different organs in the body of a mammal may differ in their metabolic rates, all together in the whole organism they maintain the weight to the $3/4$ power relation, and this is re-

flected in the relative amounts of cytochrome *c* in the animal, as well as the cytochrome oxidase content, and even the relative number of mitochondria per cell for those tissues that have been examined. While this particular example is from Schmidt-Nielsen, Calder has many more where one can see complex relations between various structures, either in their relative size or the relative rates at which they function.

These allometric properties are often interconnected so that one can be derived from another. This raises the big question: is there one principle that underlies the connections between body size and body proportions, rates of locomotion and metabolism? One original and bold insight is that of T.A. McMahon in his well-known paper (*Science* 179, 1201-1204; 1973) where he suggested that different-sized organisms may keep their elastic properties similar; and once this is achieved one can, on theoretical grounds (too lengthy to describe here), make predictions that accord well with the shapes of trees and bones, and the $3/4$ rule for metabolism, and the allometric relations measured for quadruped locomotion. This elastic similarity model is discussed by both authors and they also carefully present the empirical evidence that seems to be at variance with it. Time will tell to what extent the McMahon hypothesis prevails, but at the moment it is the most comprehensive, all-embracing explanation of this family of interconnecting allometric relations. One might assume that natural selection favours similarity of elastic properties to avoid buckling in



Some allometric generalizations, mostly for eutherian mammals. When plotted on log-log paper, the fractional exponential powers of body mass appear as the slopes of the lines. For example, body surfaces of animals, like surface areas of geometric figures, are proportional to volume (or mass, assuming constant density) raised to the $2/3$ power. On the log-log plot the $2/3$ becomes the slope, that is, an increase of 10^2 in surface when mass increases by a factor of 10^3 . (Re-drawn from a figure in *Size, Function, and Life History* (p.11), after several authors.)

larger organisms, and this could conceivably be the cause of various functional and structural size-related properties. But the question of which properties are affected by selection and which are the inevitable consequences of the internal engineering of an organism remains a difficult one.

Two points stem from the above. One is Calder's important caution that allometric interrelations are statistical correlations and they therefore cannot tell us anything about causes. The other is a point made by both authors: it may not always be the examples that come close to the allometric mean that are interesting, but the exceptions. For instance, Scholander and his colleagues showed that an arctic weasel has an exceptionally high metabolic rate, well above the norm for its size; and they suggested that because it is small, and has short legs, its fur could not be thick enough to keep it warm in winter. The weasel's solution to the problem is to stoke its internal fires to a higher rate. Another interesting case is the hearts of small shrews and hummingbirds which are larger than the expected mean. The suggestion is that were this not so their hearts would have to beat at a physically impossible rate in order to supply sufficient blood flow; they compensate by increasing their heart to a size that has a lower, but mechanically possible natural frequency. Both these examples show that selection can, no doubt within limits, alter one feature to the exclusion of the others.

The use of allometry in ecology is a new

and exciting subject, but still in its infancy. It has also been reviewed by R.H. Peters in his recent book (*The Ecological Implications of Size*, published by Cambridge University Press in 1983) in a somewhat different way from Calder, although the two books overlap to some degree. What Calder does, which is especially useful, is to consider reproduction and development, as well as population properties such as animal density and home range size, for they are all interrelated. Often information about one of these subjects provides important insights into others. This is shown particularly well in the very recent work of P.H. Harvey, R.D. Martin and others on brain size and its ecological implications, work that is in progress and therefore not reported in any of these books. But the new studies do prove that the subject is important and advancing in significant ways.

Again, the interesting questions all relate to the matter of causation. Calder is quite right to stress that what have been shown are correlations, which cannot, in the long run, be satisfying. It is like the problem of smoking and cancer; what the correlation means is the paramount issue. We must find ways of understanding what are the causes of allometric relations, and what are the causes of specific examples of deviation from those relations. This is the real strategy for the future and these two books are admirable stepping stones along the way. □

J. T. Bonner is a Professor of Biology at Princeton University.

Russian tidings

K.F. Bowden

Ocean Tides: Mathematical Models and Numerical Experiments.

By G.I. Marchuk and B.A. Kagan.
Pergamon: 1984. Pp.292. £36, \$65.

IN THE past 25 years there has been a remarkable revival of interest in ocean tides. This has been due largely to the greatly improved methods of calculation, using modern computers, but also to advances in measurement by deep-sea tide gauges and the developing use of satellite altimetry. Marchuk and Kagan are leading workers in the USSR on the theory and numerical modelling of tides. Their book is in part a monograph describing their own work and that of their colleagues, and in part a general account of the present state of knowledge. While this combination leads to some unevenness in the treatment, both aspects have something of value to offer.

The first chapter gives a clear account of the astronomical and other forces affecting the tides, the equations of tidal dynamics and general features of the methods used to solve them. The next two chapters are more mathematical and are based on the work of the authors and their colleagues on analytical and numerical procedures. While these accounts are likely to appeal mainly to practitioners in this specific field, the fourth chapter is of wider interest and comprises a survey of our present knowledge of the distribution of tides in the world ocean. Following a review of empirical charts, it summarizes the computed co-tidal and co-range charts produced in various countries and discusses a Russian version in some detail. It is interesting to note that, in their preface to the book, the authors express the view that the appearance of more advanced computers will not result in better agreement between computational and observational data. They point to the need to pay further attention to certain physical aspects of the problem.

The last two chapters deal with topics related to ocean tides but which are more specialized and again reflect Marchuk and Kagan's own research interests. In that on the benthic boundary layer, they give a comprehensive review of the subject and describe in particular a theoretical model of their own. The other is on the vertical structure of internal tidal waves which are generated mainly, in the authors' view, by the flow of a stratified ocean over an irregular sea bottom. It is estimated that, in the deep oceans, the rate of transfer of energy to internal tides is of the order of 100 times greater than the rate of dissipation by bottom friction and turbulence. □

K.F. Bowden is Emeritus Professor of Oceanography at the University of Liverpool.

Science digested

Bryan Silcock

Sci-Tech Report.

Edited by Jon Turney.

Pluto Press/Pantheon: 1984. Pp.386.
Pbk £8.95, \$11.95.

IF THE social responsibility in science movement needs a handbook, here it is: a collection of 170 shortish articles intended to 'assess the state of science and technology and locate sci-tech inventions within a wider social and political context'. The general approach might be described as left-radical-ecological. Thus nearly all of the contacts and organizations listed at the end of the book are pressure groups, most of them against something or other.

Sci-Tech Report is not a book you can sit down and read. It is far too fragmented for that. But it contains an unusual selection of information that as far as I know cannot be found elsewhere between one set of covers.

The first third, dealing with new technologies of political (in the widest sense of the word) significance — nuclear power, cable television, computers, genetic

engineering, bacteriological warfare — is fairly predictable. But we then move on to such things as accounts of science policy in the main industrialized countries and a few in the Third World as well; articles on aspects of the sociology of science such as the publishing of scientific papers, the ownership of intellectual property and fraud in science; theories of what drives the innovative process in different industries; and discussions of controversial issues, sociobiology and creationism for example, which help to form the public image of science.

The contributions range from the mainly factual to the definitely political. Their style is mostly straightforward and simple, their quality uneven. But anyone with a critical interest in science as an activity and who sees it as more than a dispassionate search for knowledge should find them well worth dipping into.

Sci-Tech Report originally appeared in France. For the English edition some material has been dropped and about 40 new articles added. This was deliberate. I assume that the dropping, in my copy at any rate, of pp. 291–306, and the duplication of pp. 307–322, was not. □

Bryan Silcock is science correspondent of the Sunday Times.

Community questions

C.R. Townsend

Ecological Communities: Conceptual Issues and the Evidence.

Edited by Donald R. Strong Jr, Daniel Simberloff, Lawrence G. Abele and Anne B. Thistle.

Princeton University Press: 1984. Pp. 613. Hbk \$60, £50; pbk \$22.50, £18.80.

ARE there rules of community assembly and generalizations to be drawn about the factors determining community composition? Or does the group of species populations that occur together at a given location result simply from stochastic processes of colonization and extinction — a random throwing together of species from the available pool? What, moreover, are the relative roles of abiotic influences and of species interactions in determining community organization? These are the central questions of community ecology.

During a period stretching from the 1940s to the 1970s, the conventional wisdom was that interspecific competition for resources dominates the evolution of co-existing species and the organization of communities. The chief proponents of this view — Lack, Hutchinson and MacArthur — considered that species interactions would often proceed according to simple Malthusian expectations, with populations growing until limited by resources and species co-existing only if they exhibit some minimal differences in their resource requirements. The view that emerges in the present volume is that the Malthusian imperative (competitive exclusion of the less efficient of species sharing resources) may often be thwarted because predation, disturbance or abiotic conditions prevent the realization of a competitive equilibrium. If competition is influential, communities are likely to be less diverse in species and, more important, the species can be expected to be more regularly strung out along the resource dimensions of the niche (because of limits to similarity) than their randomly assembled counterparts.

David Lack's work on Darwin's finches, published in 1947, can be used to illustrate several of the threads of the current debate. Lack's aim was to provide an explanation for the evolutionary diversification of *Geospiza* finches in the Galapagos archipelago from a presumed single ancestral species. Believing that the islands were similar in their habitats, he was forced to invoke interspecific competition to account for patterns in morphological characteristics (bill sizes of co-existing species overlap hardly at all) and in species distribution (some species with similar bill sizes do not co-exist). Lack interpreted such regularities as the result of competitive exclusion of similar species or evolutionary shifts generated by competition.

Lack's arguments were persuasive. But while he explained his observations and brought them together into a coherent whole, he did not test his ideas. As Grant and Schluter point out in the present book, until recently most investigators used the concept of interspecific competition both as guidance in the search for patterns and as explanation for them. What they failed to do — and here they are strongly criticized by Simberloff, Connor and Strong, among others — was to test their evidence against alternative hypotheses.

That, however, is much easier said than done. What is needed is a set of hypothetical communities, assembled in realistic ways but in which any effects of



Fitting the bill — Darwin's finches, male and female, about $\frac{2}{3}$ life-size. The illustration is taken from the facsimile edition of *Darwin's Finches* by David Lack, published last year by Cambridge University Press in their *Science Classics* series.

competition are specifically excluded. The real community can then be compared against the "neutral models" (so-called) to determine, for example, whether the naturally co-existing species are spaced more regularly along a food resource dimension of the niche than are their randomly assembled counterparts. In the case of Darwin's finches, the neutral model approach of Grant and Schluter leads to the conclusion that the competition hypothesis is, in fact, the best available. However, claims made by other workers that real communities have been structured by competition have often been called seriously into question because the actual species assemblage does not differ significantly from a hypothetical group assembled at random from the available species pool.

The construction of reasonable neutral models is not a straightforward procedure and this is cleverly demonstrated here by Colwell and Winkler. Using a computer

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program called GOD, they first generate a biota, the phylogenetic lineages of which obey specified rules. Subsets of the "mainland biota" then colonize archipelagos according to a program called WALLACE, and competition may or may not be introduced as a structuring influence. Unlike field workers, Colwell and Winkler *know* whether or not their communities are structured by competitive effects. The interesting and somewhat discomfiting conclusion is that many of the neutral model approaches used in studies of real communities often fail to distinguish any effect of competition even though competition had been included in their program. There are several reasons for this difficulty in detecting "the ghost of competition past", the most important of them being that those species most vulnerable to competitive exclusion are already likely to have been lost from the biota of the entire archipelago. The search for more appropriate neutral models is a major task for the future.

Several of the authors in *Ecological Communities*, notably Brown and Bowers, emphasize that the identification of an apparent pattern and its subsequent comparison with appropriate neutral models should only be the first step. As a result of such analysis, a causal mechanism should be suggested and subjected to experimental testing. Certainly, experiments with real communities are difficult, but they have often helped to determine whether competition (discussed in detail by Brown and Bowers, among others), predation (Dayton), facilitation (Seifert) or stochastic processes (Sale) may

be influencing community structure.

Another message that emerges clearly from the book is that some communities are far from being saturated with species — this alone will limit the general importance of competitive interactions. Both Lawton, in his important work on the insect communities on bracken, and Price, who considers fish parasites, demonstrate the occurrence of "empty niches" in communities.

Robert May, in the opening chapter, questions whether the various themes and approaches are leading us towards a resolution of the factors determining community structure. There is still a long way to go, but *Ecological Communities* must be considered to be a significant landmark on that road. A consensus is emerging (despite the rather vituperative exchanges, recorded here, between Gilpin and Diamond on the one hand and Connor and Simberloff on the other) and the book signals the general acceptance of the need to look critically at the nature of the evidence at our disposal and to give equal weight to the testing of all conceivable hypotheses, not just the one we happen to favour. At present, no one should claim too much. But we can look forward to a gradual emergence of understanding of the types of communities and environments in which particular species interactions are influential, and those in which such interactions are weak, intermittent or non-existent but where, instead, disturbance and stochastic processes predominate. □

C.R. Townsend is a Lecturer in the School of Biological Sciences, University of East Anglia.

Counsel for chemists

Robert M. Joyce

Guide to the Chemical Industry: Technology, R & D, Marketing, and Employment.

By William S. Emerson.

Wiley: 1984. Pp.330. £35, \$40.25.

Problem Solving in the Chemical Industry.

By R.J. Casey and M.J. Frazer.

Pitman: 1984. Pp.122. Pbk £8.95.

How does a chemistry student assess career opportunities in industry? Few members of university faculties have had enough industrial experience to be helpful, and friends who have recently gone to industry will have seen only a small part of the picture.

In his career counselling guide, W.S. Emerson describes what the chemical industry does and how, the roles of chemists and chemical engineers in industrial research and development, and the career opportunities that may occur. Although it addresses primarily only the development, manufacture and marketing

of organic chemicals, the general principles concerned are applicable to any part of the chemical industry. And while the reader should be able to understand organic structures in order to follow much of the discussion, the style is easy to read and the few errors are not significant.

The author begins with a tour through the principal types of products manufactured in the organic chemical industry, with descriptions of their syntheses, and then moves sequentially through the research discovery, the identification of a potential new product or process, its development and commercialization. The types of industrial research — offensive and defensive — are described, emphasizing that research produces scientific knowledge, not products or processes.

Students should learn that the development of a laboratory discovery is not just a matter of turning it over to some engineers who will simply scale up the procedure. In the 1930s, E.K. Bolton, director of Du Pont's Central Research Department, decided that the nylon his organization would develop must be 66, which could be based on plentiful benzene, rather than 510, which needed castor oil as

a raw material. In doing so, he ignored the opinion of his technical experts that 66 was too intractable, and told them to find out how to make and spin it. They did — fortunately for Du Pont, because IG Farbenindustrie was close behind with 6 nylon, which could be made from benzene. The *Guide* provides case-histories of several developments that were similarly influenced by the technical and economic decisions that must constantly be made as a project proceeds.

Chapters on patents and on unit operations introduce students to subjects that are rarely covered in chemistry curricula. A section on management and marketing gives them glimpses of typical company organizations and of the activities of managers and marketing people, the latter very briefly. This section includes a chapter on reports, which is simply descriptive; one wishes that here the author had taken the opportunity to drive home to students the critical importance of good writing.

The description of career opportunities runs the gamut, with one to two paragraphs on each. And finally, how to get a job. As Emerson points out, selling oneself is the secret to getting a job and to advancing in the organization. In all the author has done well in meeting his aims, and his *Guide* will be worth the time of any college chemistry student who wants to learn something about the chemical industry.

The same cannot be said of *Problem Solving in the Chemical Industry*. After an extended review of the philosophy of problem solving (which includes no chemistry), eight case-histories of chemical problems are presented. The emphasis is on technique: definition of the problem, exploration of various approaches, discarding unpromising ones and arriving at a solution.

However, some of the solutions only define further problems. The case-history on electrowinning of uranium covers in considerable detail seven years of the development of a pilot plant process that was then shelved for economic reasons, leaving one to wonder whether the problem to be solved had been adequately defined. The solution to the problem of disposing of dichromate-sulphuric acid cleaning solution is to me self-evident without considering the alternatives discussed. There are no exercises or problems for the student, and altogether it is rather hard to see who would benefit from reading this book. □

Robert M. Joyce, now retired, was formerly a Research Director for E.I. du Pont de Nemours, Wilmington, Delaware.

● The two volumes in the Scientific American Library reviewed in *Nature* on 15 November (*Constructing the Universe* and *The Second Law*) are in Britain generally available through bookshops. Publisher is W. H. Freeman, prices are £15.95 and £16.50 respectively.

Mathematics

Analysis of Ordinal Categorical Data. By ALAN AGRESTI. Wiley: 1984. Pp. 287. ISBN 0-471-89055-3. £34.65.

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Application of monoclonal antibodies

This week's product review concentrates on monoclonal antibodies, their production and their applications. New products include a novel gel entrapment technique as an alternative to conventional mass culture.

● Magnetic Microspheres from Molecular Biosystems, Inc. are 1–6 μm diameter spheres of denatured albumin and magnetite coated with protein A. After incubation with an antibody the spheres are used to selectively remove cells or other antigenic species from solution. The Microspheres can be used for either positive or negative selection.

Circle No. 100 on Reader Service Card.

● *Current References Using the ABC Immunoperoxidase Procedure-2*, is the second in a series of reference lists compiled by Vector Laboratories. The 120 recent publications cited in this latest list include staining of paraffin and frozen sections, cytocentrifuge preparations, solid-phase enzyme immunoassays, nitrocellulose blots, and pre- and post-embedding electron microscopy using monoclonal antibodies and lectins. A key covering a range of specific applications is also included.

Circle No. 101 on Reader Service Card.

● Genzyme Corporation of Boston, Massachusetts has completed a licence agreement with Dartmouth College (signed on 27 September) to produce and sell monoclonal antibodies to interleukin-2 from a cell line developed by Dartmouth. The monoclonal anti-interleukin-2 joins Genzyme's already extensive line of research biologicals which includes interleukin-1, interleukin-2, recombinant interleukin-2, interleukin-3, GM-colony stimulating factor and collagen 2.

Circle No. 102 on Reader Service Card.

● A panel of five new monoclonal antibodies against hepatitis B surface antigen is now available from Sera-Lab of Crawley Down, Sussex. These monoclonal antibodies, MAS 103, MAS 104, MAS 105, MAS 106 and MAS 107, have proved useful in a sandwich solid-phase radioimmunoassay for HBsAg. In particular, the use of MAS 105 as both capture and tracer antibody results in a very sensitive assay system.

Circle No. 103 on Reader Service Card.

● A new brochure, entitled *Immunochemicals and Related Products*, will be available from Boehringer Mannheim Biochemicals in January 1985. Product areas to be included in the brochure are: monoclonal antibodies; protein A products; affinity isolated antibodies; reagents for enzyme immunoassay; immunohistochemistry reagents; and cell culture products. The brochure also includes details on various immunology techniques.

Circle No. 104 on Reader Service Card.

● The Swiss company Robapharm AG of Basle has launched a range of rare monoclonal antibodies against human tissue antigens with special emphasis on cells of the reticuloendothelial system. Currently available are antibodies which distinguish vascular endothelial cells of different organs (such as lung versus liver), subtypes of tissue macrophages (resident versus inflammatory) and which are specific for smooth muscle cells. The antibodies represent new tools for research on the vascular endothelium and on mononuclear phagocytes and may be useful in the diagnosis of endothelial cell tumours, pathological changes of the vessel wall (atherosclerosis) and various cell infiltrates.

Circle No. 105 on Reader Service Card.

● The Immunex Corporation of Seattle has entered into a licence agreement with Becton Dickinson & Company for the sale of research and diagnostic products based on a monoclonal antibody developed by Immunex scientists. Becton Dickinson introduced the first products based on the antibody in late September. The agreement covers an antibody that quantifies the number of activated T-cells — antibody to interleukin-2 receptor. Three products are available based on the antibody, a purified form and two fluorescent conjugates.

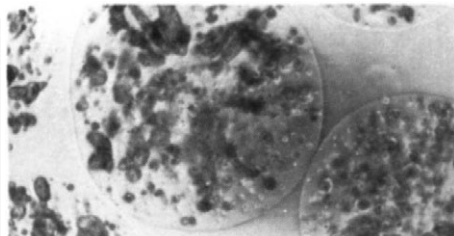
Circle No. 106 on Reader Service Card.

● The UK company, Tissue Culture Services Limited has signed an agreement with Ventrex Laboratories, Inc. of Portland, Maine, giving TCS manufacturing and distribution rights to HL-1, a serum-free cell culture media product, in either liquid or powder form, for the culture of hybridomas and certain other cell types. Use of HL-1 with a completely defined composition and extremely low protein content (less than $50\mu\text{g } \mu\text{ml}^{-1}$) facilitates the *in vitro* scale-up of monoclonal antibody production, and simplifies or eliminates purification steps.

Circle No. 107 on Reader Service Card.

● Myxothiazol, a potent inhibitor of the mitochondrial respiratory chain, is now available from Boehringer Mannheim Biochemicals. This highly toxic antibiotic is isolated from the gliding bacterium, *Myxococcus fulvus*. Myxothiazol is similar to antimycin A, in that both inhibitors bind to cytochrome *b*. However, current evidence suggests that myxothiazol binds to the (*b*₇) haem centre, while antimycin A binds to the other form (*b*_K). Acting together, these inhibitors will completely block electron transfer to cytochrome *b*.

Circle No. 108 on Reader Service Card.



The 1-mm diameter beads of the Geltrap system for monoclonal production.

● The new Geltrap system from Karyon Technology, Inc. makes possible the large-scale production of monoclonal antibodies and other mammalian and plant cell products. The gel entrapment process offers a simple, flexible and cost-effective alternative to mouse or conventional mass culture techniques. Other applications of the Geltrap system include production of hormones and alkaloids, enzyme and bacterial immobilization, and sustained release therapy.

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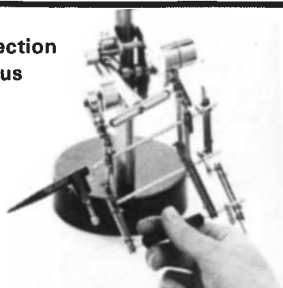
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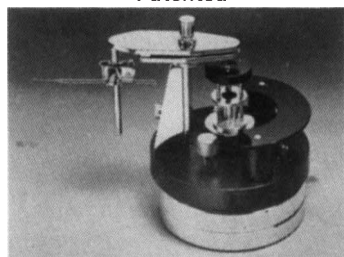
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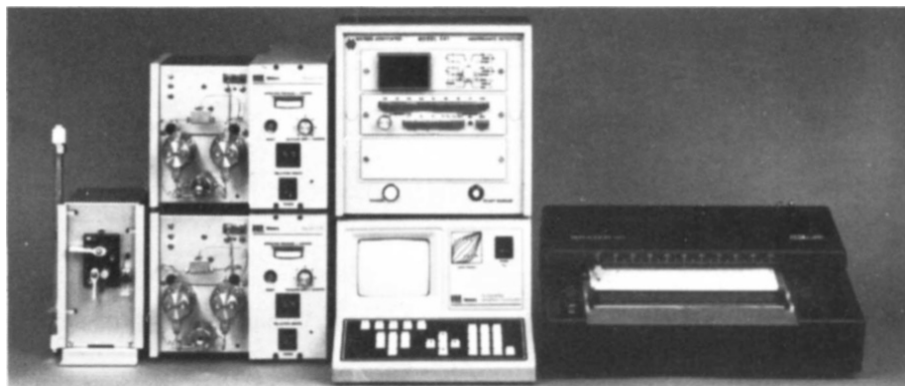
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Monoclonal antibodies can be isolated within 30 minutes on this HPLC system from Waters.

● An HPLC system designed to isolate monoclonal antibodies from ascitic fluids and cell culture supernatants has been introduced by the Waters Chromatography Division of Millipore Corporation. The HPLC system uses high-performance anion exchange chromatography to separate the monoclonal antibody from the primary constituents of ascites and cell culture supernatants — albumin, transferrin and host immunoglobins. After sample preparation using microfiltration (for ascites) or ultrafiltration (for tissue culture), the separation is typically performed in less than 30 minutes. To monitor the performance of the isolation procedure, the same HPLC system can be used to assay the homogeneity of fractions by gel filtration. The system can be easily upgraded for automated sample injection.

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● Miles Scientific now offers four monoclonal antibodies to human IgG heavy-chain determinants. These undiluted antisera offer maximum flexibility for those laboratories that want to further purify the antibody for conjugation to enzyme, fluorescence or radioisotope labels for immobilization on a solid-phase support. The products are characterized in sensitive EIA assays and show broad reactivity to a large group of purified normal and myeloma IgGs of all four subclasses.

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● Sanbio b.v. of the Netherlands, working in cooperation with the University of Leiden and other Dutch institutes, has produced a large range of monoclonal antibodies for indirect immunoperoxidase staining in tissues and ELISA techniques. The range includes anti-human keratin, neurofilament, hCG, FSH, and glia fibrillary acidic protein. Anti-human desmin, vimentin, thyroglobulin and blood vessel endothelium are also available.

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● A newly available monoclonal antibody radioimmunoassay kit for human gamma interferon provides a convenient and reliable assay method. The first product to be launched by IMRX Corporation, a joint venture of Centocor, Inc. and FMC Corporation, the IMRX Interferon-Gamma RIA is a two-step "sandwich" solid-phase radioimmunoassay.

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● Serotec Ltd is a specialist supplier of monoclonal antibodies and polyclonal antisera and has just introduced a series of 150 new products. The new range includes: sheep antisera to human, rat and mouse immunoglobulins conjugated to glucose oxidase which reacts to give a deep blue stain in immunohistology and in ELISA, and beta-galactosidase, including second antibodies; monoclonal antibodies against human proteins, such as T-helper lymphocytes, T-suppressor lymphocytes, sub/cytotoxic T cells and B lymphocytes; and monoclonal antibodies against rat proteins such as MHC I antigens and MHC II antigens (1a).

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● The Nottingham-based company Hybritech Europe offers a range of isotopic and non-isotopic kits for the measurement of human beta chorionic gonadotropin (hCG) in urine or serum. Tandem-R hCG utilizes two distinct monoclonal antibodies in a solid-phase immunoradiometric assay. Tandem-E hCG is a non-isotopic solid-phase immunoenzymetric assay for hCG using two distinct monoclonal antibodies.

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● Monoclonal antibodies against 4-azido-2-nitrophenyl (NAP) hapten have been introduced by Sera-Lab. These antibodies, MAS 089 and MA 090, are of value in the study of immunoregulation. NAP hapten is a photolabile label and can be attached covalently to the antigen binding sites of these antibodies.

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● Becton Dickinson Immunocytometry Systems has announced what is claimed to be the first comprehensive research panel for lymphoma subtyping. The panel consists of six well characterized monoclonal antibodies plus accessory reagents for immunoperoxidase staining of frozen tissue sections. The monoclonal reagents include: anti-leukocyte (a pan-leukocyte marker), multi-clone anti-T cell, anti-LEU-14 (B cells), anti-IgM, anti-kappa and anti-lambda.

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These notes are based on information provided by the manufacturers. For further details circle the appropriate numbers on the Reader Service Card bound inside the journal.